Remarks

Claims 1-4, 13-15, and 25-30 are pending and under consideration. With this Amendment, claims 1-4, 13-15, and 25-30 are being cancelled, without prejudice against their reintroduction into this or one or more timely filed continuation, divisional or continuation-in-part applications, and claims 31-47 are being newly added. Thus, after entry of this Amendment, claims 31-47 are pending and under consideration. The amendments of the claims and the various rejections raised in the Office Action are discussed in more detail, below.

I. The Amendments of the Claims

Claims 1-4, 13-15, and 25-30 have been replaced with claims 31-47.

Claim 31 is supported in the specification on page 3, lines 13-24; and page 6, lines 2-4.

Claims 32 and 33 are supported in the specification on page 3, lines 21-24; and page 19, lines 26-31.

Claim 34 is supported in the specification on page 3, lines 18-20; and page 12, lines 2-6.

Claims 35 and 36 are supported throughout the specification, such as on page 3, line 32 extending through to page 4, line 3.

Claim 37 is supported in the specification on page 4, lines 9-11; and page 16, line 31 extending through to page 16, line 16.

Claim 38 is supported in the specification page 3, lines 13-24; and page 6, lines 25-27.

Claim 39 is supported throughout the specification, such as on page 3, line 27 extending through to page 4, line 3.

Claims 40 and 41 are supported throughout the specification, such as on page 3, lines 13-27; page 6, lines 2-4; and page 12, lines 2-19.

Claims 42 and 43 are supported throughout the specification, such as on page 3, lines 21-24; and page 19, lines 26-31.

Claims 44-46 are supported throughout the specification, such as on page 3, line 32 extending through to page 4, 9-11; and page 16, line 31 extending through to page 16, line 16.

Claim 47 is supported throughout the specification, such as on page 3, line 27 extending through to page 4, line 3.

No new matter is added by virtue of the amendments. Entry of the amendments is therefore respectfully requested.

II. Rejections Under 35 U.S.C. § 112, first paragraph, written description

Claims 1-4, 13-15 and 25-30 stand rejected under 35 U.S.C. § 112, first paragraph as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. Specifically, the Patent Office contends that the specification does not describe the properties the antisense oligonucleotides must possess and that further experimentation would be required to determine the targeting sequences for the full scope of cytochrome p450 enzymes. Applicant respectfully traverses the rejection as applied to claims 31-47.

A. Legal Standard for Written Description

The standard for determining compliance with the written description requirement is whether "the description clearly allow persons of skill in the art to recognize that he or she invented what is claimed." *See In re Gosteli*, 872 F.2d 1008, 1012, 10 USPQ2d 1614, 1618 (Fed. Cir. 1991); *see also* M.P.E.P. § 2163.02. An applicant shows

possession of the claimed invention by describing the claimed invention with all of its limitations using such descriptive means as words, structures, figures, diagrams and formulas that fully set forth the claimed invention. *See Lockwood v. American Airlines, Inc.*, 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997).

Factors to be considered in determining whether there is sufficient evidence of possession include the level of skill and knowledge in the art, complete or partial structure, physical and/or chemical properties, functional characteristics alone or coupled with a known or disclosed correlation between structure and function, and the method of making the claimed invention. Disclosure of any combination of such identifying characteristics that distinguish the claimed invention from other materials and would lead one of skill in the art to the conclusion that the applicant was in possession of the claimed species is sufficient. *See* M.P.E.P. § 2163; *see also Univ. of Rochester v. G.D.Searle & Co.*, 358 F.3d 916, 69 USPQ2d 1886, 1894-5 (Fed. Cir. 2004).

However, what is conventional or well known to one of ordinary skill in the art need not be disclosed in detail. *See Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d at 1367,1384, 231 USPQ 81, 94; *see also Capon v. Eshhar*, 418 F.3d 1349, 1357, 76 USPQ2d 1078, 1085 (Fed. Cir. 2005) ("The 'written description' requirement must be applied in the context of the particular invention and the state of the knowledge. . . As each field evolves, the balance also evolves between what is known and what is added by each inventive contribution.").

B. Claims Satisfy the Written Description Requirement

In rejecting the claims, the Patent Office contends the specification does not provide sufficient guidance to allow a person skilled in the art to predict the structures of the antisense oligonucleotides possessing the property of reducing expression of a drug-metabolizing cytochrome p450 enzyme. The Patent Office asserts that the specification does not describe the broad genus of antisense oligonucleotides with the exception of the specific sequences of SEQ ID NO:18-20, 23-25, 35-36 and 46-47, emphasizing that the specification does not provide a "clear correlation between

nucleotide sequence structure, and the ability of the recited antisense oligonucleotides" to produce the desired effect.

However, knowledge of the nucleic acid sequence of the drug-metabolizing cytochrome p450 gene and corresponding transcript provides sufficient structural and functional information for a person of skill in the art to identity and design antisense oligonucleotides with the desired activity. As reiterated in prior responses, the specification and the claims clearly point out that the antisense oligonucleotides can target the AUG translation start site, the intron-exon boundary, or an exon-intron boundary. *See*, *e.g.*, *Specification*, page 3, lines 21-24, and page 19, lines 26-31. A person of skill in the art would readily recognize the regions of the nucleic acids having the stated characteristics such that the structural features (e.g., sequence) of the antisense oligonucleotides would be readily apparent to the skilled artisan if such gene sequences are available. In this regard, the specification describes various mammalian, including human, drug-metabolizing cytochrome p450 genes that were known in the art at the priority date of the instant application. These genes are described by art recognized designations, e.g., CYP1A1, CYP1A2, CYP2A6, CYP2B1, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4. For instance, the specification states:

> As noted above, sequence for numerous rat and human p450 genes are known and available to those skilled in the art through sources such as Genbank and review articles such as Gonzales 1989, Black et al., 1987, and Nelson et al. 1993 cited above. For example, Nelson et al. lists all database accession numbers for p450 genes that were available in Genbank/EMBL, SwissProt, and NBRF-PIR databases as of December 1992. . . . Since the publication of the 1993 article, other human sequences, such as those for CYP-1B1 and CYP-2B1, have also been made available in GenBank.

See Specification, page 18, line 34 extending through to page 19. Clearly, the gene sequences of a large number of drug-metabolizing mammalian cytochrome p450

enzymes were available in the art well before the priority date of the instant application. Along with the gene information, the specification also describes the classes of drugs metabolized by the exemplary cytochrome p450 enzymes, such as those for CYP1A1, CYP1A2, CYP2A6, CYP2B1, CTP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A1, CYP3A2, CYP3A4, and CYP6A1. *See Specification*, pages 15-18. Given the availability of the mammalian cytochrome p450 genes in the art and the guidance in the specification regarding the target sequences for affecting expression of the targeted enzyme, a person skilled in the art would have readily recognized the sequences and features of the morpholino antisense oligomers of the claims. As such, exemplary antisense oligonucleotides directed to different portions of the corresponding cytochrome p450 genes, such as the AUG translation start site and the intron-exon and exon-intron boundary are disclosed in the specification, as acknowledged by the Patent Office. Thus, in light of the specific description of "morpholino antisense oligomers" in the specification, the knowledge in the art of genes encoding drug-metabolizing cytochrome p450 enzymes, and the substantial understanding within the antisense field of designing antisense oligonucleotides, it is submitted that the specification provides sufficient written description for the full scope of the claims.

To illustrate Applicant's position, the Patent Office is directed to the Board of Patent Appeals and Interferences decision in *Ex parte Gleave*, Appeal No. 2005 2447, Decision on Appeal, concerning antisense technology, a copy of which is submitted herewith as Exhibit A. Although the decision is not precedential, the Board has cited the case as being "Informative." The involved application, US application Serial No. 09/619,908, is directed to use of inhibitors of IGFBP-5 expression to delay progression of hormone regulated tumor cells to an androgen-independent state. Of relevance to the rejections discussed herein are the following representative claims:

> 1. A method for delaying progression of hormone regulated mammalian tumor cells to an androgen independent state, comprising treating hormone-sensitive mammalian tumor cells with an antisense oligonucleotide which inhibits expression of IGFBP-5 by the tumor cells.

> 22. A composition for treatment of hormone-regulated cancer comprising an antisense oligonucleotide which inhibits expression of IGFBP-5 by hormone regulated tumor cells.

See Ex parte Gleave at page 4. According to the Board's decision, the specification had set forth sequences of the mouse and human IGFBP-5s along with a number of antisense sequences targeting specific regions of the mouse and human IGFBP-5 DNAs. The examiner had rejected claims 1 and 22 for insufficiency of written description, the Board summarizing the substance of the examiner's basis for the rejection as follows:

> The examiner argues that "[t]he specification only describes two target IGFBP-5 sequences, [mouse and human] . . . , and does not describe any additional sequences that can be targeted via antisense oligos. . . . Without such a description, the skilled artisan would not be able to envision any other target sequences and thus would not be able to synthesize an antisense oligo specific for the sequence" and moreover, would be required to undertake de novo experimentation to isolate and identity IGFBP-5 encoding nucleic acids.

See id at page 11. In reversing the written description rejection by the examiner, the Board stated:

> [W]e disagree with the examiner's rationale and conclusion. These claims require antisense oligonucleotides of varying scope, which inhibit expression of IGFBP-5 in hormone regulated mammalian tumor cells. . . . The examiners rationale would seem to limit the claimed genus to only those antisense oligonucleotides explicitly recited, without explaining why one skilled in the art would not have expected the mouse and human DNAs to be representative of, or have considerable structural similarity to, DNA encoding IGFBP-5 in other mammals. . . . Again, it is the

> examiner's initial burden [to] present[ ] evidence or reasons why persons skilled in the art would not recognize in the disclosure a written description of the invention defined by the claims. . . . We find that the examiner has not done so.

See id at page 12. The Board's decision is significant for its finding of written description compliance for the genus of antisense oligonucleotides targeting IGFBP-5 even though only the sequences of mouse and human forms were disclosed in the specification along with a few exemplary antisense oligonucleotides. As emphasized by the Board, the description of antisense oligonucleotides was not limited to those explicitly disclosed in the specification. Paralleling the facts in *Ex parte Gleave*, the instant application has not only disclosed specific antisense oligonucleotides, but also described that the sequences for numerous mammalian drug-metabolizing cytochrome p450 enzymes were available to the skilled artisan at the priority date.

The Board's decision in *Ex parte Gleave* and its applicability to the instant case follows Federal Circuit decisions pertaining to sufficiency of written description in situations where the structural information (e.g., sequences) of biomolecules are publically available. *See Falkner v. Inglis,* 448 F.3d 1357, 79 USPQ2d 1001 (Fed. Cir. 2005); *see also Capon v. Eshhar*, 418 F.3d 1349, 1357, 76 USPQ2d 1078, 1085 (Fed. Cir. 2005); *see also Invitrogen Corp. v. Clontech Laboratories, Inc.*, 429 F.3d 1052, 77 USPQ2d 1161 (Fed. Cir. 2005). In curtailing the rigid application of written description requirement when biomolecular sequence information is available to the skilled artisan, the Federal Circuit has stated:

> [It] is the binding precedent of this court that *Eli Lilly does not* set forth a *per se* rule that whenever a claim limitation is directed to a macromolecular sequence, the specification must always recite the gene or sequence, *regardless of whether it is known in the prior art*. . . Thus, "[w]hen the prior art includes the nucleotide information, precedent does not set a per se rule that the information must be determined afresh." . . . Indeed, a requirement that patentees recite

> known DNA structures, if one existed, would serve no goal of the written description requirement. . . .

See Falkner v. Inglis, 448 F.3d 1357, 79 USPQ2d 1001 (Fed. Cir. 2005) (emphasis added); *see also Capon v. Eshhar*, 418 F.3d 1349, 1357, 76 USPQ2d 1078, 1085 (Fed. Cir. 2005).

A decision pertinent to the instant application is *Falkner v. Inglis,* 448 F.3d 1357, 79 USPQ2d 1001 (Fed. Cir. 2005), cited above, which concerned an interference involving claims encompassing a poxvirus vaccine, where the claim recited use of poxvirus with its "essential genes" deleted or inactivated. The Board of Patent Appeals and Interferences denied a motion by the junior party (Falkner) that the senior party's (Inglis) specification did not provide sufficient written description of the essential genes of the poxvirus. The Inglis application had three passages discussing poxvirus, but discussed the identity of the essential genes and their deletion only for the herpesvirus. The Inglis specification did not provide any such detail for the poxvirus, and instead, gave a general statement that the "invention can be applied to any virus where one or more essential genes can be identified and deleted from or inactivated within the virus genome." *See id* at 1364. Despite the lack of sequences for the "essential genes" of the poxvirus and absence of any examples in the specification where such genes could be deleted or otherwise inactivated, the Federal Circuit affirmed the Board's decision of adequate written description in the Inglis specification for poxvirus "essential genes" and thus adequate written description for the claimed poxvirus vaccine. The court noted the evidence submitted by the senior party establishing that articles describing essential genes for poxvirus were well-known in the art and that the skilled person would have been readily able to choose an essential vaccinia gene "based on references that have been publicly available." *See id.* at 1366 In view of the facts of the case, the court stated:

(1) examples are not necessary to support the adequacy of written description;

(2) the written description standard may be met even where actual reduction to practice of an invention is absent; and

(3) there is no per se rule that an adequate written description of an invention that involves a biological macromolecule must contain recitation of a known structure.

See id. at 1366. The court noted "where, as in this case, accessible literature sources clearly provided, as of the relevant date, genes and their nucleotide sequences (here "essential genes"), satisfaction of the written description requirement does not require either recitation or incorporation by reference (where permitted) of such genes and sequences." *See id.*

The court reached a similar conclusion in *Invitrogen Corp. v. Clontech Laboratories, Inc.*, 429 F.3d 1052, 77 USPQ2d 1161 (Fed. Cir. 2005), which involved claims directed to a reverse transcriptase (RT) enzyme lacking RNase H activity. The specification provided *a sequence* of a deleted form of the enzyme from Murine Maloney Leukemia Virus, whereas the claims covered a broad genus of modified RT enzymes, including those derived from "a retrovirus, yeast, Neurospora, Drosophila, primates, and rodents." *See Invitrogen Corp. v. Clontech Laboratories, Inc.*, 429 F.3d at 1074. The infringer asserted that the claims to the modified enzyme were invalid under the written description clause of 35 U.S.C § 112, first paragraph because the claims at issue were not limited to sequence of RT genes recited in the specification. The Federal Circuit, however, upheld the district court's determination of adequate written description, noting the lower court's determination that "at the time of the invention, sequence of RT genes were known and member of the RT gene family shared significant homologies from one species of RT to another and that the sequence for the claimed modified enzymes and *other representative RT genes* were known by the critical date. *See id.* at 1073. Even though sequences of mutant enzymes for the other reverse transcriptases had not been described in the specification, the court emphasized the knowledge available to the public of the sequences of other RT enzymes, such as enzymes from viruses HTLV-1, BLV, RSV, and HIV. The Federal Circuit concluded:

> [T]he shared written description for the patents-in-issue recites both the DNA and amino acid sequences of a

> *representative embodiment* of the claimed RT enzyme. The specification also discloses test data that the enzyme produced by the listed sequence has the claimed features-DNA polymerase activity without RNase H activity. Under both the *Eli Lilly* and *Fiers* analysis, the specification at bar is sufficient.

See Invitrogen Corp. v. Clontech Laboratories, Inc., 429 F.3d at 1073 (emphasis added). In both *Falkner v. Inglis* and *Invitrogen Corp. v. Clontech Laboratories,* the court found adequate written description for biomolecules where a *representative number* of species were known in the art, even when the specification disclosed only a single embodiment of the claimed genus.

Applicant points out that, as shown by the art of record, cytochrome p450 enzymes are a structurally related class of proteins, both by sequence and by having a heme cofactor, comprising a protein superfamily involved in metabolic transformation reactions. The instant specification has described a subset of these enzymes having the functional property of metabolic transformation of pharmaceutical compounds, and that the gene sequences of a significant and representative number of such drug-metabolizing enzymes were known to the skilled artisan at the priority date of this application. Illustrations of their common structure are clearly set forth by references to publications describing the cytochrome p450 family and in the specification through description of the percent amino acid sequence similarities of relevant functional domains of one such cytochrome p450 subgenus of the CYP3A enzymes. *See Specification*, page 14, lines 1-38. As emphasized herein, a person skilled in the art of antisense field would have readily recognized the antisense sequences for use in the claimed subject matter based on the available knowledge of the gene sequences for drug-metabolizing cytochrome p450 enzymes. In light of the guidance for determining sufficiency of written description delineated by the Board of Patent Appeals and Interferences and by the decisions of the Federal Circuit discussed herein, Applicant has amply satisfied the written description clause of 35 U.S.C. § 112, first paragraph.

Reconsideration and withdrawal of the rejection for insufficiency of written description is respectfully requested.

III. Rejections Under 35 U.S.C. § 112, first paragraph, enablement

Claims 1-4, 13-15, and 25-30 stand rejected under 35 U.S.C. § 112, first paragraph, because the specification allegedly does not enable any person skilled in the art to which it pertains, or with which it is most connected to make and use the invention commensurate in scope with the claims. Applicant respectfully traverses the rejection as applied to claims 31-47.

A. Legal Standard for Enablement

The standard for determining enablement under first paragraph of 35 U.S.C. §112 is whether the specification enables any person skilled in the art to which it pertains to make and use the claimed invention without undue experimentation. *See, e.g., In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991); *see also United States v. Telectronics, Inc.*, 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988) ("The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation."). A patent need not teach, and preferably omits, what is well known in the art. *See In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *see also* M.P.E.P. § 2164.01.

Moreover, a considerable amount of experimentation is permissible if it is merely routine, or if the specification provides a reasonable amount of guidance with respect to the direction in which experimentation should proceed. *See Ex parte Forman*, 230 USPQ 546, 547 (BPAI 1986); *see also In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988). The fact that the required experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. *See In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988).

B. Enablement is Commensurate with the Scope of the Claims

As an initial matter, the Patent Office acknowledges the enablement of the claimed method for antisense morpholino oligomers of SEQ ID NO:18-20, 23-25, 35-36, and 46-47. *See Office Action*, page 5. The cyctochrome p450 enzymes targeted by these antisense oligomers include CYP2E1, CYP3A2, and CYP3A4. Nevertheless, the Patent Office contends the specification does not provide a level of enablement commensurate with the scope of the claims. Specifically, the rejection states:

> No guidance or working examples are disclosed that would allow a skilled artisan to use any other antisense oligonucleotides to *inhibit all forms of cytochrome p450* in vitro or in vivo, nor does the specification as filed teach that the above antisense oligonucleotides can be used to inhibit the *metabolism of all drug metabolizing p450 enzymes*. . . . Applicants have not provide a clear correlation between the ability of the specified antisense oligonucleotides . . . to inhibit activity of specific variants of cytochrome p450 enzymes and their ability to *inhibit all known variants* of the cytochrome p450.

See Office Action, page 7 (emphasis added). As part of the rejection, the Patent Office contends that antisense technology is an unpredictable art such that undue experimentation would be required to identify other antisense oligonucleotides capable of having the desired effect of reducing expression of a drug metabolizing cytochrome p450 enzyme, pointing to a number of antisense oligonucleotides (i.e., SEQ ID NO:16-17, 21-22, and 37-38) tested which allegedly did not affect expression of the target enzyme. *See Office Action*, page 8.

However, the scope of enablement must bear only a "reasonable correlation" to the scope of the claims. *See* M.P.E.P. § 2164.08; *see*, *e.g.*, *In re Angstadt*, 537 F.2d 498, 190 USPQ 214 (CCPA 1976). Requirement for description of antisense oligonucleotides for "all forms of cytochrome p450" or a showing of inhibition of the

metabolism of "all drug metabolizing p450 enzymes" goes beyond the "reasonable correlation" standard, as discussed in the decision of *In re Angstadt*:

> Appellants have apparently not disclosed every catalyst which will work; they have apparently not disclosed every catalyst which will not work. The question then is whether in an unpredictable art, section 112 requires disclosure of a test with every species covered by a claim. To require such a complete disclosure would apparently necessitate a patent application or applications with "thousands" of examples or the disclosure of "thousands" of catalysts along with information as to whether each exhibits catalytic behavior resulting in the production of hydroxyperoxides. More importantly, such a requirement would force an inventor seeking adequate patent protection to carry out a prohibitive number of actual experiments.

See In re Angstadt, 537 F.2d at 502. As such, the court held that "appellants are not required to disclose every species encompassed by their claims" even in an unpredictable art to satisfy the enablement requirement. *See id.* at 503; *see also Chiron Corp. v. Genentech*, Inc., 363 F.3d 1247, 70 USPQ2d 1321 (Fed. Cir. 2004) *citing AK Steel Corp. v. Sollac*, 344 F.3d 1234, 1244 (Fed. Cir. 2003) ("That is not to say that the specification itself must necessarily describe how to make and use every possible variant of the claimed invention, for the artisan's knowledge of the prior art and routine experimentation can often fill gaps, interpolate between embodiments, and perhaps even extrapolate beyond the disclosed embodiments, depending upon the predictability of the art."). Moreover, in determining the scope of enablement, the Patent Office must consider the state of the art, including the knowledge available to the skilled artisan:

> The scope of enablement . . . is that which is disclosed in the specification *plus the scope of what would be known to one of ordinary skill in the art* without undue experimentation.

See Invitrogen Corp. v. Clontech Laboratories, Inc., 429 F.3d 1052, 77 USPQ2d 1161 (Fed. Cir. 2005).

As noted above for the instant application, the art of record and the descriptions in the specification show that a significant number of drug-metabolizing mammalian cytochrome p450 gene sequences, including members of different classes of drug-metabolizing enzymes, were available to the skilled artisan at the priority date of the instant application. *See Specification*, page 18, line 31 to page 19, line 11. For designing morpholino antisense oligonucleotides targeting such enzymes, the specification also provides specific guidance to the skilled artisan for blocking (e.g., inhibiting) expression of the targeted drug-metabolizing cytochrome p450 enzyme, guidance of which including the oligomer length, the structure of the intersubunit linkages, the oligomer T_m, and importantly, the target regions:

> Exemplary oligomer sequences can be designed according to the following guidelines: . . . Each oligomer either (a) spans the AUG start codon of the indicated gene, with the CAU complement of the start codon (expressed in a 5' to 3' direction) being positioned near the center or near the 3' end of the oligomer, or (b) spans an intron-exon (splice donor) boundary or, more preferably, an exon-intron (splice acceptor) boundary of an unspliced pre-mRNA sequence . . .

See Specification, page 19, lines 25-34. The specification also provides exemplary antisense oligonucleotides, such as the sequences recited on page 20 (see Table 2). Although the Patent Office asserts the field of antisense technology is unpredictable, Applicant has presented working examples of specific antisense oligonucleotides targeted to various regions of the processed and preprocessed transcript, including AUG translation start sites as well as splice junctions. In each example, Applicant has identified antisense oligonucleotides capable of blocking expression of the targeted cytochrome p450 enzyme. Thus, the specification provides ample guidance and direction in which experimentation should proceed for identifying other morpholino antisense oligomers capable of blocking expression of a drug-metabolizing mammalian cytochrome p450 enzyme, including other enzymes not specifically tested in the specification.

Nevertheless, the Patent Office in support of unpredictability in the art has pointed to a number of antisense oligonucleotides (i.e., SEQ ID NOS:16-17, 21-22, and 37-38) that allegedly did not reduce expression of the targeted enzyme. However, SEQ ID NO:16 was effective in blocking phenobarbitol induced CYP2B1 expression (as evidenced by sleep time following hexobarbitol administration) while SEQ ID NO:17 is targeted to a region of the CYP21B mRNA at base 855, which is removed from the AUG start codon. *See Specification*, page 27, lines 26-27. In addition, the two oligonucleotides, SEQ ID NOS: 37 and 38, had 2 bases purposely reversed to create mismatches (80% identity with target sequence), which lowers the melting temperature of the oligonucleotide. Hence, the tests with the oligos of SEQ ID NOS: 17, 37 and 38, rather than showing unpredictability, provides guidance on the antisense sequences to be designed and used for targeting the selected cytochrome p450 enzyme. The sequences of SEQ ID NOS: 37 and 38 also demonstrate the specificity of the antisense oligonucleotides that were effective in blocking expression, since a search of GenBank with the sequences of SEQ ID NO:37 and 38 did not show homology to any sequence in GenBank. *See Specification*, page 25, lines 20-23. Furthermore, while attempting to point out some morpholino antisense oligomers that allegedly did not affect expression of the targeted enzyme, the Patent Office has not proffered any rationale as to why the guidance on the design and testing of morpholino antisense oligomer that in fact reduced expression of the targeted cytochrome p450 enzyme could not be extrapolated to any other such enzyme. Although some experimentation may be required to identity morpholino antisense oligonucleotides for other drug-metabolizing cytochrome p450 genes, the level of experimentation required is not undue, particularly given the description and guidance provided in the specification and the high level of skill in the art.

As part of the enablement rejection, the Patent Office dismisses the references and declarations presented by Applicant in the response filed August 8, 2006. The references of Zhang et al., 2006, *Antiviral Res.*, Zhang et al., 2006, *Veterinary Microbiol.*, and Opalinska et al., 2006, Science STKE, are considered post-filing publications that allegedly attempt to enable the claimed subject matter while the

declarations of Dwight Weller and Patrick Iversen are considered not relevant to the full scope of the claimed subject matter. However, the references are offered by Applicant to show the level of skill in the art regarding the use of morpholino antisense oligomers at the priority date of the instant application. *See Gould v. Quigg*, 822 F.2d 1074, 1077, 3 USPQ2d 1302, 1305 (Fed. Cir. 1987) ("[I]t is true that a later dated publication cannot supplement an insufficient disclosure in a prior date application to render it enabling. In this case, the later dated publication was not offered as evidence for this purpose. Rather it was offered as evidence of the level ordinary skill in the art at the time of the application and as evidence that the disclosed device would have been operative."). As for the declarations, they are offered to show that *in vivo* administration of morpholino antisense oligomers and targeting of morpholino antisense oligomers to AUG start sites and splice junctions to affect metabolism of a drug (e.g., Paclitaxil, which is taxol) were operative at the filing date of the application, confirming the descriptions in the specification (*see, e.g., Specification*, page 17, line 14, and Example 5).

In all, when the specification and working examples are properly viewed, they provide ample guidance to the skilled artisan on the design of antisense oligonucleotides targeted to various regions of a drug-metabolizing mammalian cyctochrome p450 gene to inhibit expression of the enzyme and affect metabolism of a drug metabolized by the enzyme. It is submitted that the descriptions in the specification, including the working examples, counterbalance any general unpredictability in the field of antisense technology that might bear on the claimed subject matter.

In support of the enablement of the instant claims for its full scope, Applicant again directs the Patent Office to the Board of Patent Appeals and Inferences decision in *Ex parte Gleave*, discussed above. In *Ex parte Gleave*, the examiner had rejected the relevant claims recited above for lack of enablement as well as for insufficient written description. The examiner's basis of the enablement rejection is summarized by the Board as follows:

> According to the examiner, the claims are drawn to "antisense oligo[nucleotides] targeted to any transcript of IGFBP-5 as well as methods of treatment using the antisense oligonucleotides] . . . but the specification is "only enabling for antisense oligos of SEQ ID NO:1 targeted to the IGFBP-5 transcripts of [murine] SEQ ID NO:`13, and for the use of SEQ ID NOS:2, 3 and 9 in the inhibition of SEQ ID NO:14 in vitro, and does not provide guidance on the in vivo inhibition of [human] SEQ ID NO:14" (id.). . . According to the examiner, the "clinical application of antisense therapy is a highly unpredictable art due to obstacles that still face antisense therapy" (Answer, page 9). The obstacles enumerated by the examiner are essentially: the identification of an appropriate target in the disease process, the identification of a molecule that can interfere with the disease process; the identification of a molecule that can interfere with the disease process through specific recognition and affinity; the complexity of cellular uptake of oligonucleotides; and physical barriers due to internal structures of target RNAs and associations with cellular proteins. Id., pages 9-10. In addition, the examiner relies on Gerwtiz and Branch as evidence that the "the antisense approach has generated controversy [among those of skill in the art] with regard to mechanism of action, reliability, and ultimate therapeutic utility" (id., page 10), and the sense in the art is that "efforts should be increased . . . to learn how they may be used successfully in the clinic" (id.).

See Ex parte Gleave at pages 15 and 17. The Board reversed the rejection by the examiner, directly addressing the arguments on unpredictability in the field:

> We have no reason to doubt the examiner's assessment of the state of the art in general, and we think it is fair to say that the field of antisense therapy is indeed recognized as highly unpredictable by those skill in the art. Nevertheless, applellents point out, and the examiner appears to

> acknowledge, that appellants have identified the murine and human IGFBP-5s as appropriate targets in treating androgen-dependent cancers like prostate cancer and breast cancer, and that appellents have identified antisense IGFBP-5 molecules that can delay the progression to androgen independence in the Shionogi tumor model (asserted to be useful model of human prostate cancer) and/or inhibit expression of IGFBP-5 in human prostate cancer cell lines.

See id. at 17 and 18. After weighing the various factors used for assessing sufficiency of enablement, the Board concluded as follows:

> This concrete guidance, in the form of working examples addresses a number of the examiner's specific concerns, and weigh in favor of finding the specification enabling for claims directed to antisense inhibition of IGFBP-5. In any case, the examiner has not explained why the specific guidance in the specification would not, at least to some extent, mitigate or counter balance any remaining factors (e.g., the generally unpredictable nature of the field) tending to weigh against the finding of enablement.

See id. at 18. It is of note that the facts and the basis of the examiner's enablement rejection in *Ex Part Gleave* parallel those in the instant case.

The Board's decision in *Ex parte Gleave* is in accord with the Federal Circuit holding in *Falkner v. Inglis,* which has been extensively discussed above. In addition to the written description issue, the junior party Falkner asserted that the Inglis' specification did not enable the claimed poxvirus vaccine since the specification did not provide any working examples of poxvirus deleted of "essential genes" or a description of essential genes of poxvirus nor provide any specific guidance on construction of the poxvirus vaccine. However, the Federal Circuit upheld the decision of the Board of Patent Appeals and Interferences, which concluded the specification sufficiently enabled the full scope of the claims. The court emphasized: (a) the publication in

professional journals of the DNA sequence of the poxvirus genome along with locations of the "essential regions", (b) the high skill of those in the art; and (c) the well known differences between poxvirus and herpesvirus, such that it would have aided a person skilled in the art to apply the lesson of the herpesvirus example to the poxvirus. The court reiterated the Board's observation:

> The mere fact that the experimentation may have been difficult and time consuming does not mandate a conclusion that such experimentation would have been considered to be "undue" in this art.

See Falkner v. Inglis, 448 F.3d at 1365. Applicant points out that poxvirus is not a single type of virus, but comprises a large family of DNA viruses that replicate in the cytoplasm, and as suggested by Exhibit B (Gubser et al., 2004, "Poxvirus genomes: a phylogenetic analysis," *J. Gen. Virol.* 85:105-117), not all poxviruses had been sequenced by the priority date of the Inglis application (Ser. No. 08/459,040 filed September 25, 1990), and yet the Board of Patent Appeals and Interferences, as confirmed by the Federal Circuit , held the Inglis specification enabled for the full scope of the claims to poxvirus vaccines.

A similar outcome is presented in *Invitrogen Corp. v. Clontech Laboratories, Inc.*, 429 F.3d 1052, 77 USPQ2d 1161 (Fed. Cir. 2005), which has also been discussed above. The claims in *Invitrogen Corp. v. Clontech Laboratories, Inc*. encompassed compositions of a modified reverse transcriptase enzyme lacking RNase activity. The claims encompassed the genus of such modified enzymes, where the unmodified enzyme could be from a variety of organisms, including "retrovirus, yeast, Neurospora, Drosophila, primates, and rodents." *See id.* at 1071. The working example was limited to generation of the modified (i.e., deletion mutant) form based on the enzyme obtained from Maloney Murine Leukemia Virus. Despite the limited disclosure, the Federal Circuit affirmed the lower court's ruling that the specification enabled the full scope of the claims. *See id.* at 1071.

In view of the evidence as a whole, and in accord with decisions by the Board of Patent Appeals and Interferences and the Federal Circuit, the present specification sufficiently enables a person of skill in the art to which it pertains to make and use the claimed invention without undue experimentation for the full scope of the claims. Accordingly, reconsideration and withdrawal of the rejections under the enablement clause of 35 U.S.C. § 112, first paragraph is respectfully requested.

IV. Obviousness Double-Patenting Rejection

Claims 1-4, 13-15, and 25-30 stand rejected under the judicially created doctrine of obviousness-type double patenting over claims 1-20 of U.S. Patent No. 6,686,338. In the response filed August 8, 2006, Applicant has submitted a terminal disclaimer with respect to co-owned US Patent No. 6,673,778. Applicant will submit in a separate filing a Terminal Disclaimer with respect to the obviousness-type double patenting rejection over US Patent No. 6,686,388. Applicant respectfully requests consideration and withdrawal of the obviousness-type double patenting rejection upon submission of such terminal disclaimer.

V. Conclusion

It is submitted in view of the foregoing that all of the substantive rejections as applied to the pending claims are overcome and that the claims are in condition for allowance upon submission of an appropriate terminal disclaimer. If the Examiner believes that any unresolved issues are better addressed by a conference, the Examiner is encouraged to contact the undersigned representative at Tel. No. 650.838.4365.

No fees beyond those submitted herewith are believed to be due in connection with this Amendment. However, the Director is authorized to charge any additional fees

that may be required, or credit any overpayment, to Perkins Coie LLP Deposit Account No. 50-2207 (Order No. **50450-8311.US03**).

Respectfully submitted,

Date: October 29, 2007

/ Euk Oh /

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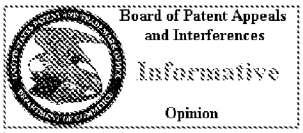
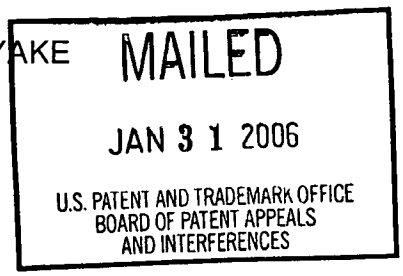
EXHIBIT A



The opinion in support of the decision being entered today was not written for publication and is not binding precedent of the Board.

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES



Ex parte MARTIN GLEAVE and HIDEAKI MIYAKE

Appeal No. 2005-2447
Application No. 09/619,908

HEARD: October 18, 2005

Before SCHEINER, ADAMS and MILLS, Administrative Patent Judges.

SCHEINER, Administrative Patent Judge.

DECISION ON APPEAL

This is a decision on appeal under 35 U.S.C. § 134 from the final rejection of claims 1-23 and 26-40. Claims 24 and 25, also pending in the application, have been allowed.

BACKGROUND

> Insulin-like growth factor (IGF)-I and IGF-II are potent mitogens for many normal and malignant cells. Accumulating evidence suggests that IGFs play an important role in the pathophysiology of prostatic disease and breast cancer. . . .
>
> The biological response to [IGFs] is regulated by various factors, including IGFBPs [(insulin-like growth factor binding proteins)]. To date, six IGFBPs have been identified whose function is believed to involve modulation of the biological actions of the IGFs through high affinity interactions . . . However, some evidence suggests biological activity for IGFBPs that are independent of IGFs, . . . and both stimulatory and inhibitory effects of IGFBPs on cell proliferation have been reported under various experimental conditions. . . .

Specification, pages 1-2.

"[P]rostate cancer is an androgen-sensitive tumor, [thus,] androgen withdrawal . . . is utilized in some therapeutic regimens . . . [and] leads to extensive apoptosis in the prostate tumor, and hence to a regression of the disease. However, . . . apoptosis is not complete, and a progression of surviving tumor cells to androgen-independence ultimately occurs." Id., page 1. The present invention is concerned with delaying the ultimate progression of tumor cells to androgen-independence.

Appellants "initially characterized the changes [in] IGFBPs expression in the Shionogi tumor model[1] after castration and during [progression to androgen-independence]" (Specification, page 5). "Of the IGBFPs expressed in Shionogi tumors, the most dramatic changes in expression were observed with IGFBP-5. Despite undetectable levels in [androgen-dependent] intact tumors, IGFBP-5 expression is highly upregulated after castration, and remains highly expressed in [androgen-independent] tumors." Id., pages 5-6. Moreover, "[t]he pattern of

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[1] "The Shionogi tumor model is a xenograft of an androgen-dependent mouse mammary carcinoma that grows subcutaneously in male syngenic hosts." Specification, pages 4-5. Shionogi tumor cells "are highly tumorigenic and locally invasive . . . [and] have been shown to respond to androgen withdrawal in a manner which mimics the observed behavior of prostatic tumor cells," that is, "androgen withdrawal precipitates apoptosis and tumor regression in a highly reproducible manner" (id., page 5). "Further, changes in expression of peptides . . . in human prostate cancer following castration and during progression to androgen-independence are similar to those observed in Shionogi tumor cells. Because of these similarities, the Shionogi tumor model mimics human prostate cancer and provides a very useful model for the evaluation of the ability of compounds to delay the onset of androgen-independence. Despite complete tumor regression after castration, rapidly growing androgen-independent Shionogi tumors invariably recur after one month, which provides a reliable end point to evaluate agents which can delay the progression to androgen-independence." Id.

IGFBP-5 upregulation in the Shionogi tumor model during [progression to androgen-independence] . . . is similar to that in rat prostate . . . and human prostate" (id., page 6).

According to appellants, antisense oligodeoxynucleotides (ODNs) complementary to portions of the gene encoding IGFBP-5 "inhibit[ ] cell proliferation and induce[ ] cell cycle arrest in Shionogi tumor cells in a time- and dose-dependent manner . . . [and do] not appear to induce apoptosis either in vitro or in vivo, . . . suggest[ing] that antisense IGFBP-5 activity occurs via inhibition of cell proliferation rather than induction of apoptosis." Id. Appellants "hypothesized that targeting upregulation precipitated by androgen using [an] antisense strategy might inhibit progression to androgen-independence." Id., page 7. In appellants' "in vivo experiments, administration of antisense IGFBP-5 after castration delayed time to [androgen-independence] . . . and inhibited [androgen-independent] recurrent tumor growth." Id.

THE CLAIMS

The present invention is directed to "a method for delaying the progression of hormone-regulated (prostatic or breast) tumor cells to hormone (e.g. androgen or estrogen) independence, a therapeutic method for the treatment of individuals . . . suffering from hormone regulated cancers, such as breast or prostate cancer, and therapeutic agents effective for use in such methods." Specification, page 4. In addition, the present invention is directed to a method of inhibiting or delaying metastatic boney progression of an IGF-1 sensitive tumor in a mammal. We note that the claims on appeal require an antisense oligonucleotide that inhibits expression of

IGFBP-5, with the exception of method claims 8, 12, 15, 19, 39 and 40, which merely require "a composition effective to inhibit expression of IGFBP-5."

Claims 1, 8, 15 and 22 are representative of the subject matter on appeal:

1. A method for delaying progression of hormone-regulated mammalian tumor cells to an androgen-independent state, comprising treating hormone-sensitive mammalian tumor cells with an antisense oligonucleotide which inhibits expression of IGFBP-5 by the tumor cells.

8. A method for treating a hormone-responsive cancer in a mammalian individual suffering from hormone-responsive cancer, comprising the steps of initiating hormone-withdrawal to induce apoptotic cell death of hormone-responsive cancer cells in the individual, and administering to the individual a composition effective to inhibit expression of IGFBP-5 by the hormone-responsive cancer cells, thereby delaying the progression of hormone-responsive cancer cells to a hormone-independent state in the individual.

15. A method for inhibiting or delaying metastatic boney progression of an IGF-1 sensitive tumor in a mammal, comprising the step of administering to the mammal a composition effective to inhibit expression of IGFBP-5 by the hormone-responsive cancer cells, thereby inhibiting or delaying metastatic boney progression of the tumor.

22. A composition for treatment of hormone-regulated cancer comprising an antisense oligonucleotide which inhibits expression of IGFBP-5 by hormone-regulated tumor cells.

THE REJECTIONS

The claims stand rejected as follows:

I. Claims 1, 5, 22 and 36-38[2] under 35 U.S.C. § 102 (b) as anticipated by Huynh.[3]

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[2] Claims 36-38 were subject to this ground of rejection in the final rejection (paper no. 14, January 24, 2003), but were omitted from the examiner's statement of the rejection in the Answer. The omission of these claims appears to have been a typographical error, as they are specifically discussed in the examiner's response to appellants' arguments (see, e.g., page 16 of the Answer).

[3] Huynh et al., "A Role for Insulin-like Growth Factor Binding Protein 5 in the Antiproliferative Action of the Antiestrogen ICI 182780," Cell Growth & Differentiation, Vol. 7, pp. 1501-1506 (November 1996)

II. Claims 1-3, 5, 6, 22, 23, 26-28, and 36-38[4] under 35 U.S.C. § 103 (a) as unpatentable over Huynh in view of Kiefer,[5] Baracchini[6] and Nickerson.[7]

III. Claims 1-3, 4, 6, 8-10, 12, 13, 15-17, 19, 20, 22, 23 and 38-40 under the first paragraph of 35 U.S.C. § 112, written description.

IV. Claims 1-23 and 26-40 under the first paragraph of 35 U.S.C. § 112, enablement.

DISCUSSION

I. Anticipation

Claims 1, 5, 22 and 36-38 stand rejected under 35 U.S.C. § 102 (b) as anticipated by Huynh. Claims 1, 5 and 38 are method claims, while claims 22, 36 and 37 are composition claims. Appellants argue that the method and composition claims do not stand or fall together because "anticipation of a method claim requires a different content of the reference than a composition claim, which need only disclose the same composition, rather than the same method steps." Brief, page 3. Accordingly, we will consider claim 1 to be representative of the method claims, and claim 22 to be representative of the composition claims – claims 5 and 38 will stand or fall with claim 1, while claims 36 and 37 will stand or fall with claim 22.

Claim 1 is directed to a method of delaying progression of hormone-regulated

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[4] Claim 40 was included in this rejection in the final rejection, but the rejection was withdrawn with respect to claim 40 in the Examiner's Answer (page 17).

[5] Kiefer et al., "Molecular Cloning of a New Human Insulin-like Growth Factor Binding Protein," Biochem. Biophys. Res. Commun., Vol. 176, No. 1, pp. 219-225 (1991).

[6] U.S. Patent No. 5,801,154, issued to Baracchini et al. on September 1, 1998.

[7] Nickerson et al., "Castration-Induced Apoptosis in the Rat Ventral Prostate is Associated with Increased Expression of Genes Encoding Insulin-Like Growth Factor Binding Proteins 2, 3, 4 and 5," Endocrinology, Vol. 139, No. 2, pp. 807-810 (1998).

mammalian tumor cells to an androgen-independent state by treating the cells with an antisense oligonucleotide which inhibits expression of IGFBP-5 by the tumor cells. According to the examiner, "a key limitation is that the method steps are carried out in hormone sensitive mammalian tumor cells" (Answer, page 14), and "Huynh discloses administering an antisense oligomer comprising 21 nucleotides targeted to IGFBP-5 to breast cancer cells" (id., page 5). The examiner acknowledges that Huynh says nothing about delaying progression of hormone-regulated mammalian tumor cells to an androgen-independent state, but argues that "any recited outcome such as that is merely considered to be an inherent feature, since all the structural and manipulative features of the claim are present in Huynh" (id.).

It is well settled that a prior art reference may anticipate even when claim limitations are not expressly found in that reference, but are nonetheless inherent in it. See, e.g., Atlas Powder Co. v. IRECO Inc., 190 F.3d 1342, 51 USPQ2d 1943 (Fed. Cir. 1999); Titanium Metals Corp. v. Banner, 778 F.2d 775, 227 USPQ 773 (Fed. Cir. 1985). However, it is also the case that "[i]nherency . . . may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient." In re Oelrich, 666 F.2d 578, 581, 212 USPQ 323, 326 (CCPA 1981).

Here, Huynh teaches that "IGFBP-5 can either stimulate or inhibit cellular proliferation in different experimental systems . . . suggest[ing] that there are poorly characterized complexities in IGFBP-5 action" (Huynh, pages 1503-1504). Indeed, on this record, there is no dispute that "Huynh [ ] actually teach[es] that antisense to IGFBP-5 stimulates cell proliferation in the [MCF-7] breast cancer cell line used" (Answer, page 14), while it inhibits proliferation in the Shionogi tumor cells used by

appellants. According to the examiner, this variation in the effects of antisense IGFBP-5 is irrelevant "because cellular proliferation (or inhibition thereof) is not recited as a claim limitation" (id.). In our view, however, this variation is relevant because it shows that in the only directly comparable parameter of record, the two cell lines react differently to inhibition of IGFBP-5. While Huynh says nothing about delayed progression to androgen-independence, it is not unreasonable to expect that the two cell lines might react differently to inhibition of IGFBP-5 in this respect as well, especially in light of Huynh's suggestion that the actions of IGFBP-5 are poorly characterized. In our view, the examiner has established that inhibition of IGFBP-5 in Huynh's MCF-7 cells might delay progression to androgen-independence, but has not established that it will. As discussed above, this is not sufficient to establish a prima facie case of anticipation based on inherency.

Accordingly, the rejection of claims 1, 5 and 38 as anticipated by Huynh is reversed.

Claim 22, however, stands on a different footing. Claim 22 is directed to "a composition for treatment of hormone-regulated cancer comprising an antisense oligonucleotide which inhibits expression of IGFBP-5 by hormone-regulated tumor cells." Huynh plainly describes an IGFBP-5 antisense oligodeoxynucleotide which reduces expression of IGFBP-5 in human breast cancer cells. Appellants argue that "the phrase 'for treatment of hormone-regulated cancer' is more than a statement of intended use and deserves to be given weight in assessing the scope of the claims." Brief, page 7. According to appellants, "Huhnh's antisense is not used in the treatment of any animal or human . . . [thus,] [t]here is no teaching of a composition suitable for

administration in the treatment of cancer." Id. Nevertheless, appellants have not pointed out anything which makes Huynh's IGFBP-5 antisense oligonucleotide composition unsuitable for administration to an animal, or which distinguishes it from the claimed IGFBP-5 antisense oligonucleotide composition in any way.

Accordingly, the rejection of claim 1 as anticipated by Huynh is affirmed. As discussed above, claims 36 and 37 stand or fall with claim 22, thus the rejection of claims 36 and 36 as anticipated by Huynh is affirmed as well.

II. Obviousness

Claims 1-3, 5, 6, 22, 23, 26-28, and 36-38 stand rejected under 35 U.S.C. § 103 (a) as unpatentable over Huynh in view of Kiefer, Baracchini and Nickerson. Having already determined that Huynh anticipates the subject matter of claims 22, 36 and 37, we affirm the rejection under 35 U.S.C. § 103 (a) with respect to those claims. "[A]nticipation is the epitome of obviousness." Connell v. Sears, Roebuck & Co., 722 F.2d 1542, 1548, 220 USPQ 193, 198 (Fed. Cir. 1983).

Claims 1-3, 5, 6, 23, 26-28 and 38, on the other hand, are directed to methods of delaying the progression of hormone-regulated tumor cells to an androgen-independent state; to treating a hormone-responsive cancer; and to delaying metastatic boney progression of IGF-1 sensitive tumors by inhibiting IGFBP-5.

The examiner relies on Huynh for disclosure of "an antisense oligomer comprising 21 nucleotides targeted to IGFBP-5 that was administered to breast cancer cells" (Answer, page 6); on Kiefer for disclosure of the translation initiation and termination regions of IGFBP-5 (id.); and on Baracchini for "teach[ing] that the translation initiation and termination regions are preferred regions for targeting with

antisense oligos" (id.). According to the examiner, these references provide motivation for targeting particular regions of IGFBP-5 in order to inhibit its effects. Id., pages 6-7.

Nevertheless, in our view, the dispositive issue here is the examiner's proposed rationale for inhibiting IGFBP-5 in tumor cells in the first place. The underlying premise of the examiner's rejection is that "Nickerson teaches that prostatic tumor cells over-express IGFBP-5 and [that IGFBP-5] is involved in tumorigenesis" (id., page 6), and that, therefore, it would have been obvious for one skilled in the art to inhibit IGFBP-5 expression in prostatic tumor cells (id., page 7).

We see no factual basis for the examiner's expansive interpretation of Nickerson's teachings. Nickerson's experiments were designed "to study the gene expression of IGFBPs during involution of the rat ventral prostate after castration." Nickerson, page 807. The experiments demonstrated that "IGFBP-5 mRNA increases in the ventral prostate 2-fold by 24 h and 5-fold by 72 h [ ] in keeping with the hypothesis that IGFBP-5 may be involved in apoptosis resulting from steroid hormone deprivation." Id., page 809, left-hand column. According to Nickerson, the experimental system could not determine "whether IGFBPs cause apoptosis in the ventral prostate or are upregulated as a result of apoptosis." Id., right-hand column. Either way, the examiner has not explained how Nickerson's observations suggest that IGFBP-5 is involved in tumorigenesis, or why one skilled in the art would have wanted to inhibit its effects.

The examiner bears the initial burden of establishing prima facie obviousness. See In re Rijckaert, 9 F.3d 1531, 1532, 28 USPQ2d 1955, 1956 (Fed. Cir. 1993). To support a prima facie conclusion of obviousness, the prior art must disclose or suggest all the limitations of the claimed invention. See In re Lowry, 32 F.3d 1579, 1582, 32

USPQ2d 1031, 1034 (Fed. Cir. 1994). In addition, the record must provide evidence that those of skill in the art would have had a reasonable expectation of success in doing so. See In re Dow Chemical Co., 837 F.2d 469, 473, 5 USPQ2d 1529, 1531 (Fed. Cir. 1988).

We agree with appellants that the examiner's rejection "fails to state a prima facie case of obviousness." Brief, page 8. The rejection of claims 1-3, 5, 6, 23, 26-28 and 38 under 35 U.S.C. § 103 is reversed.

III. Written Description

Claims 1-3, 4, 6, 8-10, 12, 13, 15-17, 19, 20, 22, 23 and 38-40 stand rejected under the first paragraph of 35 U.S.C. § 112, as lacking adequate written descriptive support.

"The 'written description' requirement serves a teaching function, . . . in which the public is given 'meaningful disclosure in exchange for being excluded from practicing the invention for a limited period of time.'" University of Rochester v. G.D. Searle & Co., Inc., 358 F.3d 916, 922, 69 USPQ2d 1886, 1891 (Fed. Cir. 2004) (citation omitted). Another "purpose of the 'written description' requirement is . . . [to] convey with reasonable clarity to those skilled in the art that, as of the filing date [ ], [the applicant] was in possession of the invention." Vas-Cath Inc. v. Mahurkar, 935 F.2d 1555, 1563-64, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991). See also Enzo Biochem Inc. v. Gen-Probe Inc., 296 F.3d 1316, 1329, 63 USPQ2d 1609, 1617 (Fed. Cir. 2002). The requirement is satisfied when the specification "set[s] forth enough detail to allow a person of ordinary skill in the art to understand what is claimed and to recognize that the inventor invented what is claimed." University of Rochester, 358 F.3d at 928, 69

USPQ2d at 1896. Whether or not a specification satisfies the requirement is a question of fact, which must be resolved on a case-by-case basis (Vas-Cath, 935 F.2d at 1562-63, 19 USPQ2d at 1116), and it is the examiner's "initial burden [to] present[ ] evidence or reasons why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims" (In re Wertheim, 541 F.2d 257, 263, 191 USPQ 90, 97 (CCPA 1976)).

With respect to claims 1-3, 4, 6, 9, 10, 13, 16, 17, 20, 22, 23 and 38, we disagree with the examiner's rationale and conclusion. These claims require antisense oligonucleotides, of varying scope, which inhibit expression of IGFBP-5 in hormone-regulated mammalian tumor cells. The examiner argues that "[t]he specification . . . only describes two target IGFBP-5 sequences, [mouse and human] . . . , and does not describe any additional sequences that can be targeted via antisense oligos. Without such a description, the skilled artisan would not be able to envision any other target sequences and thus would not be able to synthesize an antisense oligo specific for the sequence" (Answer, page 8), and moreover, would be "required to undertake de novo experimentation to isolate and identify IGFBP-5 encoding nucleic acids" (id.).

Nevertheless, "applicants have some flexibility in the 'mode selected for compliance' with the written description requirement" (University of Rochester, 358 F.3d at 928, 69 USPQ2d at 1896), and it is well settled that actual reduction to practice is not necessary to satisfy the requirement (id. at 926, 69 USPQ2d at 1894). On the other hand, "[i]n claims to genetic material . . . [a] definition by function . . . does not suffice to to define [a] genus because it is only an indication of what the [material] does, rather than what it is." University of California v. Eli Lilly and Co., 119 F.3d 1559, 1568, 43 USPQ2d 1398, 1406 (Fed. Cir. 1997). The court explained that "[a]n adequate written

description of a DNA . . . 'requires a precise definition, such as by structure, formula, chemical name, or physical properties,'" (id. at 1566, 43 USPQ2d at 1404) while "[a] description of a genus of cDNAs may be achieved by means of a recitation of a representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus or of a recitation of structural features common to the members of the genus, which features constitute a substantial portion of the genus" (id. at 1568, 43 USPQ2d at 1406). Subsequently, the court clarified that "the written description requirement would be met for [a claim] . . . if [a] functional characteristic . . . were coupled with a disclosed correlation between that function and a structure that is sufficiently known or disclosed." Enzo Biochem, 296 F.3d at 1324-25, 63 USPQ2d at 1613.

Here, the specification sets forth the sequences of DNA molecules encoding the mouse and human IGFBP-5s, as well as a number of antisense sequences targeting specific regions of the mouse and human IGFBP-5 DNAs. The examiner's rationale would seem to limit the claimed genus to only those antisense oligonucleotides explicitly recited, without explaining why one skilled in the art would not have expected the mouse and human DNAs to be representative of, or have considerable structural similarity to, DNA encoding IGFBP-5 in other mammals. Again, it is the examiner's "initial burden [to] present[ ] evidence or reasons why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims" (Wertheim, 541 F.2d at 263, 191 USPQ at 97). We find that the examiner has not done so.

Accordingly, the rejection of claims 1-3, 4, 6, 9, 10, 13, 16, 17, 20, 22, 23 and 38 as lacking adequate written descriptive support under 35 U.S.C. § 112, first paragraph, is reversed.

With respect to claims 8, 12, 15, 19, 39 and 40, however, we agree with the examiner that adequate written descriptive support is lacking. We note that these claims merely require "a composition" effective to inhibit expression of IGFBP-5. The only such compositions disclosed in the specification are the afore mentioned antisense oligonucleotides. The examiner's position is essentially that the specification does not provide "any description, structural[ ] or otherwise, of IGFBP-5 inhibitors other than the instantly described antisense oligo[nucleotides]" and that the instantly described antisense oligonucleotides are "not representative of the breadth of inhibitors sought in the instant claims" (Answer, page 8).

Appellants argue that "the invention is based on the discovery . . . that reducing the expression of IGFBP-5 in hormone-responsive cancer cells has therapeutic benefits" (Brief, page 12), and "antisense inhibitors of IGFBP-5 expression [are] examples of a methodology that can be used in practicing the methods" (id., page 13). Appellants argue that the invention "is not antisense technology per se. It is also not the identification of IGFBP-5, nor any and all inhibitors of IGFBP-5 expression" (id., page 12).

These arguments are not persuasive. The Federal Circuit has recently held that the written description standard discussed in Eli Lilly applies to methods as well as products. See University of Rochester v. G.D. Searle & Co., Inc., 358 F.3d 916, 926, 69 USPQ2d 1886, 1894 (Fed. Cir. 2004): "Regardless whether a compound is claimed per se or a method is claimed that entails the use of the compound, the inventor cannot lay claim to that subject matter unless he can provide a description of the compound sufficient to distinguish infringing compounds from non-infringing compounds, or infringing methods from non-infringing methods."

The facts in Rochester are similar to those of the instant application. Rochester involved a "method for selectively inhibiting PGHS-2 activity in a human host, comprising administering a non-steroidal compound that selectively inhibits activity of the PGHS-2 gene product to a human host in need of such treatment." Id. at 920, 69 USPQ2d at 1888 (emphasis added). The court noted that the relevant patent described the cells needed to screen for compounds having the recited property, as well as "assays for screening compounds, including peptides, polynucleotides, and small organic molecules to identify those that inhibit the expression or activity of the PGHS-2 gene product." Id. At 927, 69 USPQ2d at 1895. Nevertheless, the court concluded that the patent's disclosure was inadequate to enable the claimed method because the patent "[did] not disclose just which peptides, polynucleotides, and small organic molecules have the desired characteristic of selectively inhibiting PGHS-2." Id. (emphasis in original, internal quotations omitted). "Without such disclosure, the claimed methods cannot be said to have been described." Id.

In this case, as in Rochester, the claims are directed to a process for accomplishing a desired result (in Rochester, selectively inhibiting PGHS-2 activity in a human host; here, "inhibiting expression of IGFBP-5 in hormone-responsive cells") using a composition having a specified functional property (in Rochester, a "non-steroidal compound that selectively inhibits activity of the PGHS-2 gene product"; here, "a composition effective to inhibit expression of IGFBP-5"). And in this case, as in Rochester, the specification provides no description whatsoever of just which compositions have the functional property recited in the claims - the genus recited in the claims is defined exclusively in functional terms, i.e., in terms of what the members of the genus do, rather than what they are.

As discussed above, "[a] definition by function . . . does not suffice to define [a] genus because it is only an indication of what the [material] does, rather than what it is." Eli Lilly, 119 F.3d at 1568, 43 USPQ2d at 1406. To paraphrase Eli Lilly, naming a type of material, which may or may not exist, in the absence of knowledge as to what that material consists of, is not a description of that material. See id. Accordingly, the rejection of claims 8, 12, 15, 19, 39 and 40 as lacking adequate written descriptive support under 35 U.S.C. § 112, first paragraph, is affirmed.

IV. Enablement

Claims 1-23 and 26-40, all the claims on appeal, stand rejected under 35 U.S.C. §112, first paragraph, as lacking enablement. According to the examiner, the claims are drawn to "antisense oligo[nucleotides] targeted to any transcript of IGFBP-5 as well as methods of treatment using said antisense oligo[nucleotides]" (Answer, page 9), but the specification "is only enabling for antisense oligos of SEQ ID NO:1 targeted to the IGFBP-5 transcripts of [murine] SEQ ID NO:13, and for the use of SEQ ID NOS; 2, 3 and 9 in the inhibition of SEQ ID NO:14 in vitro, and does not provide guidance on the in vivo inhibition of [human] SEQ ID NO:14" (id.).

With respect to claims 1-7, 9-11, 13, 14, 16-18, 20-23 and 26-48, all of which require an antisense oligonucleotide capable of inhibiting expression of IGFBP-5, we do not agree with the examiner's rationale or conclusion, for the reasons that follow. Initially, however, we note that the examiner has focused exclusively on the therapeutic use of antisense oligonucleotides, and has not separately addressed the enablement of those claims that do not require antisense oligonucleotides (as was done in the written description rejection above). Nevertheless, our affirmance of the written description rejection for claims 8, 12, 15, 19, 39 and 40 constitutes a disposition of these broader

claims, so we need not reach the merits of the enablement rejection with respect to these claims.

Returning to claims 1-7, 9-11, 13, 14, 16-18, 20-23 and 26-48, then, we find that the reasons cited in support of the examiner's rejection are insufficient to support the examiner's conclusion that these claims are not enabled by the specification.

"The first paragraph of 35 U.S.C. § 112 requires, inter alia, that the specification of a patent enable any person skilled in the art to which it pertains to make and use the claimed invention. Although the statute does not say so, enablement requires that the specification teach those in the art to make and use the invention without 'undue experimentation.' In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988).[8] That some experimentation may be required is not fatal; the issue is whether the amount of experimentation is 'undue.'" In re Vaeck, 947 F.2d 488, 495, 20 USPQ2d 1438, 1444 (Fed. Cir. 1991) (emphasis in original). Nevertheless, "[w]hen rejecting a claim under the enablement requirement of section 112," it is well settled that "the PTO bears an initial burden of setting forth a reasonable explanation as to why it believes that the scope of protection provided by that claim is not adequately enabled by the description of the invention provided in the specification of the application; this includes,

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> Factors to be considered in determining whether a disclosure would require undue experimentation have been summarized by the board in Ex parte Forman [230 USPQ 546, 547 (BdPatAppInt 1986)]. They include (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims (footnote omitted).
>
> In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988).

of course, providing sufficient reasons for doubting any assertions in the specification as to the scope of enablement." In re Wright, 999 F.2d 1557, 1561, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993).

According to the examiner, "the clinical application of antisense therapy is a highly unpredictable art due to obstacles that still face antisense therapy" (Answer, page 9). The obstacles enumerated by the examiner are essentially: the identification of an appropriate target in the disease process; the identification of a molecule that can interfere with the disease process through specific recognition and affinity; the complexity of cellular uptake of oligonucleotides; and physical barriers due to internal structures of target RNAs and associations with cellular proteins. Id., pages 9-10. In addition, the examiner relies on Gewirtz[9] and Branch[10] as evidence that "'the antisense approach has generated controversy [among those of skill in the art] with regard to mechanism of action, reliability, and ultimate therapeutic utility'" (id., page 10), and the sense in the art is that "'efforts should be increased . . . to learn how they may be used successfully in the clinic'" (id.).

We have no reason to doubt the examiner's assessment of the state of the art in general, and we think it is fair to say that the field of antisense therapy is indeed recognized as highly unpredictable by those of skill in the art. Nevertheless, appellants point out, and the examiner appears to acknowledge, that appellants have identified the murine and human IGFBP-5s as appropriate targets in treating androgen-dependent cancers like prostate cancer and breast cancer, and that appellants have identified

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[9] Giwirtz et al., "Facilitating Oligonucleotide Delivery: Helping Antisense Deliver on Its Promise," Proc. Natl. Acad. Sci. USA, Vol. 93, pp. 3161-3163 (April, 1996).

[10] Branch, A.D., "A Good Antisense Molecule is Hard to Find," TIBS, Vol. 23, pp. 50 (February, 1998).

antisense IGFBP-5 molecules that can delay progression to androgen independence in the Shionogi tumor model (asserted to be a useful model of human prostate cancer) and/or inhibit expression of IGFBP-5 in human prostate cancer cell lines. See page 17 of the substitute Brief for Appellant (submitted June 10, 2004), and page 9 of the Answer. This concrete guidance, in the form of working examples, would seem to address a number of the examiner's specific concerns, and weigh in favor of finding the specification enabling for claims directed to antisense inhibition of IGFBP-5. In any case, the examiner has not explained why the specific guidance in the specification would not, at least to some extent, mitigate or counterbalance any remaining factors (e.g., the generally unpredictable nature of the field) tending to weigh against a finding of enablement. In other words, the examiner has not explained why identifying other antisense IGFBP-5 molecules capable of delaying progression of hormone-regulated tumor cells to androgen-independence, either in vivo or in vitro would have required undue experimentation, given the specific guidance provided by appellants in their working examples.

Accordingly, the rejection of claims 1-7, 9-11, 13, 14, 16-18, 20-23 and 26-48 as lacking enablement under the first paragraph of 35 U.S.C. § 112 is reversed.

SUMMARY

I. The rejection of the claims under 35 U.S.C. § 102 (b) as anticipated by Huynh is affirmed with respect to claims 22, 36 and 37, and reversed with respect to claims 1, 5 and 38.

II. The rejection of the claims under 35 U.S.C. § 103 (a) as unpatentable over Huynh, Kiefer, Baracchini and Nickerson is affirmed with respect to claims 22, 36 and 37, and reversed with respect to claims 1-3, 5, 6, 23, 26-28 and 38.

III. The rejection of the claims under 35 U.S.C. § 112, first paragraph, as lacking adequate written descriptive support is affirmed with respect to claims 8, 12, 15, 19, 39 and 40, and reversed with respect to claims 1-3, 4, 6, 9, 10, 13, 16, 17, 20, 22, 23 and 38.

IV. The rejection of the claims under 35 U.S.C. § 112, first paragraph, as lacking enablement is reversed with respect to claims 1-7, 9-11, 13, 14, 16-18, 20-23 and 26-48. We do not reach the merits of this rejection with respect to claims 8, 12, 15, 19, 39 and 40.

TIME PERIOD FOR RESPONSE

No time period for taking any subsequent action in connection with this appeal may be extended under 37 CFR § 1.136 (a).

AFFIRMED-IN-PART

Toni R. Scheiner
Administrative Patent Judge

Donald E. Adams
Administrative Patent Judge

Demetra J. Mills
Administrative Patent Judge

) BOARD OF PATENT
) APPEALS AND
) INTERFERENCES

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Poxvirus genomes: a phylogenetic analysis

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The evolutionary relationships of 26 sequenced members of the poxvirus family have been investigated by comparing their genome organization and gene content and by using DNA and protein sequences for phylogenetic analyses. The central region of the genome of chordopoxviruses (ChPVs) is highly conserved in gene content and arrangement, except for some gene inversions in *Fowlpox virus* (FPV) and species-specific gene insertions in FPV and *Molluscum contagiosum virus* (MCV). In the central region 90 genes are conserved in all ChPVs, but no gene from near the termini is conserved throughout the subfamily. Inclusion of two entomopoxvirus (EnPV) sequences reduces the number of conserved genes to 49. The EnPVs are divergent from ChPVs and between themselves. Relationships between ChPV genera were evaluated by comparing the genome size, number of unique genes, gene arrangement and phylogenetic analyses of protein sequences. Overall, genus *Avipoxvirus* is the most divergent. The next most divergent ChPV genus is *Molluscipoxvirus*, whose sole member, MCV, infects only man. The *Suipoxvirus*, *Capripoxvirus*, *Leporipoxvirus* and *Yatapoxvirus* genera cluster together, with *Suipoxvirus* and *Capripoxvirus* sharing a common ancestor, and are distinct from the genus *Orthopoxvirus* (OPV). Within the OPV genus, *Monkeypox virus*, *Ectromelia virus* and *Cowpox virus* strain Brighton Red (BR) do not group closely with any other OPV, *Variola virus* and *Camelpox virus* form a subgroup, and *Vaccinia virus* is most closely related to CPV-GRI-90. This suggests that CPV-BR and GRI-90 should be separate species.

INTRODUCTION

The poxviruses represent a family of large DNA viruses that replicate in the cytoplasm. *Variola virus* (VAR), the cause of the disease smallpox, is the most notorious poxvirus and was eradicated by vaccination with *Vaccinia virus* (VV) (Fenner *et al.*, 1988), a related *Orthopoxvirus* (OPV) of unknown origin (Baxby, 1981). The poxvirus family is subdivided into the entomopoxvirus (EnPV) and chordopoxvirus (ChPV) subfamilies (*Entomopoxvirinae* and *Chordopoxvirinae*), which infect insects and chordates, respectively (Moss, 2001). The ChPVs are further divided into eight genera (*Avipoxvirus*, *Molluscipoxvirus*, *Orthopoxvirus*, *Capripoxvirus*, *Suipoxvirus*, *Leporipoxvirus*, *Yatapoxvirus* and *Parapoxvirus*), whereas the EnPVs are divided into three genera (*A*, *B* and *C*). Although poxvirus genome organization, replication, host range and pathogenesis have been studied extensively (Moss, 2001), less is known about the evolutionary relationships of these viruses.

Recently, the number of poxvirus genome sequences has increased considerably (Table 1) (Upton *et al.*, 2003) and the sequences of two EnPVs and at least one member of each ChPV genus, except genus *Parapoxvirus*, are available for comparison. These data enable analysis of the evolutionary relationships of these viruses and the result of such an investigation is presented here. To study the evolutionary relationships of poxviruses, we have compared the size of virus genomes, the number of conserved and unique genes and their arrangement within the genome. The nucleotide or amino acid sequences of subsets of those genes were then used for phylogenetic analyses. Lastly, we have considered the OPV genus in more detail. The results showed that in addition to the close relationship of VAR and *Camelpox virus* (CMPV) that was noted previously (Gubser & Smith, 2002), *Monkeypox virus* (MPV), which causes a disease with clinical similarity to smallpox, is divergent from all OPVs, and so are *Ectromelia virus* (ECT) and *Cowpox virus* (CPV) Brighton Red (BR). Data presented also suggest that the classification of CPV-BR and CPV-GRI-90 as two strains of the same species should be reassessed.

METHODS

DNA sequence comparison from central genomic regions. The central ~100 kb of 11 OPVs (98 872 nucleotides in VV Copenhagen, VV-COP) were aligned with the program CLUSTALW version 1.8 (Thompson *et al.*, 1994) using the default parameters.

Table 1. Poxvirus complete genomic sequences

Poxvirus complete genomic sequences. The A+T contents are as described by authors or have been calculated using the program Composition from the GCG package. ITRs, Inverted terminal repeats.

Genus	Species		Strain	Genome (bp)	A+T (%)	ITRs (kbp)	Reference	GenBank accession no
Chordopoxviruses								
Orthopoxvirus	*Vaccinia virus*	VV	Copenhagen	191 636	66·6	12·0	Goebel *et al.* (1990)	M35027
			MVA	177 923	66·6	9·8	Antoine *et al.* (1998)	U94848
			Tian Tan	189 274	66·8	7·5	–	AF095689
	Variola virus	VAR	Bangladesh-1975	186 102	66·3	0·7	Massung *et al.* (1994)	L22579
			India-1967	185 578	67·3		Shchelkunov *et al.* (1995)	X69198
			Garcia-1966	186 986	67·3	0·6	Shchelkunov *et al.* (2000)	Y16780
	Monkeypox virus	MPV	Zaire-96-I-16	196 858	68·9	6·4	Shchelkunov *et al.* (2001)	AF380138
	Ectromelia virus	ECT	Moscow	209 771	66·8		–	AF012825
			Naval	207 620	66·9	7·4	–	*
	Camelpox virus	CMPV	CM-S	202 185	66·9	6	Gubser & Smith (2002)	AY009089
			M-96	205 719	66·8	7·7	Afonso *et al.* (2002b)	AF438165
	Cowpox virus	CPV	Brighton Red	224 501	66·6		–	AF482758
Leporipoxvirus	*Myxoma virus*	MYX	Lausanne	161 774	56·4	11·5	Cameron *et al.* (1999)	AF170726
	Shope fibroma virus	SFV	Kaza	159 857	60·5	12·4	Willer *et al.* (1999)	AF170722
Avipoxvirus	*Fowlpox virus*	FPV		288 539	69·0	9·5	Afonso *et al.* (2000)	AF198100
Capripoxvirus	*Lumpy skin disease virus*	LSDV	Neethling 2490	150 733	73·0	2·4	Tulman *et al.* (2001)	AF325528
	Goatpox virus	GTPV	Pellor	149 935	75·0	2·3	Tulman *et al.* (2002)	AY077835
			G20-LKV	149 695	75·0	2·2	Tulman *et al.* (2002)	AY077836
	Sheeppox virus	SPPV	TU-V02127	149 995	75·0	2·2	Tulman *et al.* (2002)	AY077832
			Strain A	150 057	75·0	2·3	Tulman *et al.* (2002)	AY077833
			NISKHI	149 662	75·0	2·1	Tulman *et al.* (2002)	AY077834
Suipoxvirus	*Swinepox virus*	SWPV	17077-99	146 454	72·0	3·7	Afonso *et al.* (2002a)	AF410153
Molluscipoxvirus	*Molluscum contagiosum virus*	MCV	Subtype 1	190 289	36·0	4·7	Senkevich *et al.* (1996)	U60315
Yatapoxvirus	*Yaba-like disease virus*	YLDV		144 575	73·0	1·9	Lee *et al.* (2001)	AJ293568
Entomopoxvirus								
EnPV B	*Melanoplus sanguinipes*	EnPVm		236 120	81·7	7	Afonso *et al.* (1999)	AF063866
	Amsacta moorei	EnPVa		232 392	81·5	9·4	Bawden *et al.* (2000)	AF250284

*ECT-NAV sequence data were produced at the Sanger Institute in collaboration with Antonio Alcamí of the Department of Pathology, University of Cambridge and can be obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/ev/.

The nucleotide coordinates of the aligned sequences are: VV modified virus Ankara (VV-MVA, 30791–129436; Antoine *et al.*, 1998); VV-COP (38938–137809; Goebel *et al.*, 1990); VAR-India-1970 (VAR-IND, 26672–125468; Shchelkunov *et al.*, 1995); VAR-Garcia-1966 (VAR-GAR, 27657–126473; Shchelkunov *et al.*, 2000); VAR-Bangladesh-1975 (VAR-BSH, 27296–126098; Massung *et al.*, 1994); CMPV-M-96 (38694–137667; Afonso *et al.*, 2002b); CMPV-CMS (36904–135787; Gubser & Smith, 2002); MPV (36079–134701; Shchelkunov *et al.*, 2001); ECT-Naval (ECT-NAV, 42379–141280; www.sanger.ac.uk); and CPV-BR (52492–151512; accession no. AF482758). The transition/transversion ratio was estimated at 2·72 using the program Treepuzzle (Strimmer & von Haeseler, 1996) and this value was used for the construction of a maximum-likelihood distance matrix using the DNADIST program from the PHYLIP package version 3.6 (alpha2) (Felsenstein, 1989), with the F84 model of nucleotide substitution (Felsenstein, 1984).

Alignment of multiple protein sequences. Amino acid sequences of individual proteins were aligned by a method similar to that used previously (McGeoch *et al.*, 2000). Sequences of each protein from the different viruses were aligned separately by each of the programs CLUSTALW (Thompson *et al.*, 1994), Dialign2 (Morgenstern, 1999) and Multalin (Corpet, 1988) using the default program parameters. For each set of data, combined alignments were produced by re-extracting the individual sequences from these three alignments, with retention of the gapping characters introduced by each program. Then a new alignment was made from this triple set of sequences using the program CLUSTALW. All positions in the combined alignment that had a gap in any sequence were then excised, thus deleting both unanimously placed gaps and sections where the three primary alignments were in conflict.

Phylogenetic analysis of multiple protein sequences. The amino sequences of (i) 17 proteins that are conserved in all ChPVs or (ii) 12 proteins that are present in 12 OPVs were aligned individually and positions with gaps were excluded from the alignments as described above. The individual alignments were concatenated to form a single file of 10 451 (i) and 2316 (ii) amino acids, respectively. The most appropriate model of sequence evolution was determined

using the program Treepuzzle. For both concatenated alignments, neighbour-joining trees (Saitou & Nei, 1987) were constructed using the programs Prodist and Neighbor from the PHYLIP package version 3.0 (Felsenstein, 1989). These were then used as starting trees to construct the maximum-likelihood trees (Felsenstein, 1973) using the program ProML according to the Jones–Taylor–Thornton model (Jones *et al.*, 1992) with gamma distribution. Bootstrap analyses (Felsenstein, 1985) were performed on both trees using the programs SEQBOOT (1000 random replicates, random number seed = 133333), Protdist and CONSENSE.

Phylogenetic analysis of multiple OPV DNA sequences. The nucleotide sequences of 12 genes present in the terminal region of 12 OPVs were aligned individually with the program CLUSTALW version 1.8 (Thompson *et al.*, 1994). Positions with gaps were excluded from the alignment by manual inspection and individual alignments were concatenated to form a single file of 7233 (all genes), 4170 (genes present in the left end of the genome) and 3063 (genes present in the right end of the genome) nucleotides. For all concatenated alignments, neighbour-joining trees were constructed using the program PAUP* (Swofford, 2003) and these were used as starting trees for the construction of maximum-likelihood trees (Felsenstein, 1973) implemented using PAUP*. The model of nucleotide substitution used, as determined with Modeltest (Posada & Crandall, 1998), was the General Time Reversible (GTR) model with gamma distribution and proportion of invariable sites (shape parameter of the gamma distribution = 0·7082; proportion of invariable sites = 0·5676). The robustness of trees was evaluated by bootstrap analysis of the neighbour-joining trees, with 1000 rounds of replication, using PAUP*.

Similarity analysis. An analysis of the similarity of the terminal region of the CPV-BR and ECT-NAV genomes was done on the concatenated alignment used for phylogenetic alignment of OPV DNA sequences by using the program SimPlot (Ray, 1997). Genetic similarity was calculated according to the F84 model of evolution with a transition/transversion rate of 1·95.

RESULTS

Poxvirus gene content and genome organization

The sequences of 26 poxviruses have been determined (Table 1) and these include a member of each ChPV genus, except genus *Parapoxvirus*, and EnPVs *A* and *C*. A comparison showed that the general organization of the ChPV genome is conserved, with the central region (Fig. 1) encoding very similar proteins for RNA and DNA synthesis, protein processing, virion assembly and structural proteins. In contrast, genes encoded by terminal regions are more divergent between different ChPV genera, species within a genus and even strains of the same species. Many of these genes are non-essential for virus growth *in vitro*, and encode proteins affecting host range, virulence or interaction with the host immune system (Moss, 2001). Despite these similarities in ChPV genomes, the length varies from an estimated 139 kb in *Orf virus* to 289 kb in *Fowlpox virus* (FPV) and the A + T content varies from 75 % in the genus *Capripoxvirus* to 36 % in the genus *Parapoxvirus* (Moss, 2001) (Table 1). In contrast, EnPV genomes are more divergent and the gene order differs from ChPVs and between different EnPV genera (Afonso *et al.*, 1999; Bawden *et al.*, 2000).

Comparison of sequenced poxviruses identified 90 genes that are conserved in all ChPVs and this number is reduced to 49 by including two EnPVs (Table 2), consistent with a recent report (Upton *et al.*, 2003). Amongst ChPVs, these 90 conserved genes are all located within the central 100 kb region of the genome (Fig. 1). In contrast, no gene in the terminal region of any ChPV is conserved throughout the subfamily. If the sequence of VV strain Tian Tan (TT) (accession no. AF095689) is also included, the number of conserved genes drops to 80 due to gene fragmentation. The ten genes (*F12L*, *F15L*, *G6R*, *G8R*, *L5R*, *H4L*, *D4R*, *D11L*, *A4L*, *A28L* – named using VV-COP nomenclature) that are broken in VV-TT but conserved in all other ChPVs encode proteins that are essential for virus transcription (H4L, G8R and D11L), or form part of the intracellular mature virus (IMV) core (A4L), or are required for virus dissemination (F12L) (van Eijl *et al.*, 2002). These observations suggest strongly that there are sequencing errors in the VV-TT genome sequence. Consistent with this view, an alignment of the central 100 kb of 11 OPV genomes (VV-COP, VV-MVA, VV-TT, MPV-Zaire, VAR-BSH, VAR-IND, VAR-GAR, CMPV-CM-S, CMPV-M-96, ECT-NAV and CPV-BR) revealed many genes that contained multiple positions at which the sequence of 10 OPVs is identical but at which VV-TT differed (see supplementary data at JGV Online: http://vir.sgmjournals.org). For these reasons the sequence of VV-TT is excluded from most analyses. Recently, Upton *et al.* (2003) also reported errors in the VV-TT genome and demonstrated by resequencing that many of the genes reported to be disrupted were in fact complete.

An alignment of the central region of the genome from viruses from seven ChPV genera [VV, *Myxoma virus* (MYX), *Yaba-like disease virus* (YLDV), *Lumpy skin disease virus* (LSDV), *Swinepox virus* (SWPV), FPV and *Molluscum contagiosum virus* (MCV)] is shown in Fig. 1. The VV genome is taken as the reference against which others are compared and each VV gene is shown as a vertical bar. Genes that are conserved in all ChPVs (Table 2) are shown in grey. Genes shown in black are conserved in at least two ChPV genera. For viruses from other genera, only genes that differ from VV are illustrated and genes unique to individual viruses are shown in colour. This shows that, in this region of the genome, the *OPV*, *Leporipoxvirus*, *Yatapoxvirus*, *Suipoxvirus* and *Capripoxvirus* genera contain only 3, 2, 1, 1 and 0 unique genes, respectively, indicating that these viruses are closely related. In contrast, MCV and FPV encode 40 or 33 unique genes within the central part of their genome, suggesting that these viruses are more divergent.

Fig. 1 also demonstrates that in the central region of ChPV genomes, the overall gene order and content are very well conserved between the the OPV, *Leporipoxvirus*, *Yatapoxvirus*, *Capripoxvirus*, *Suipoxvirus* and *Molluscipoxvirus* genera. A notable feature is the presence of a gene (*C7L* in VV-COP,

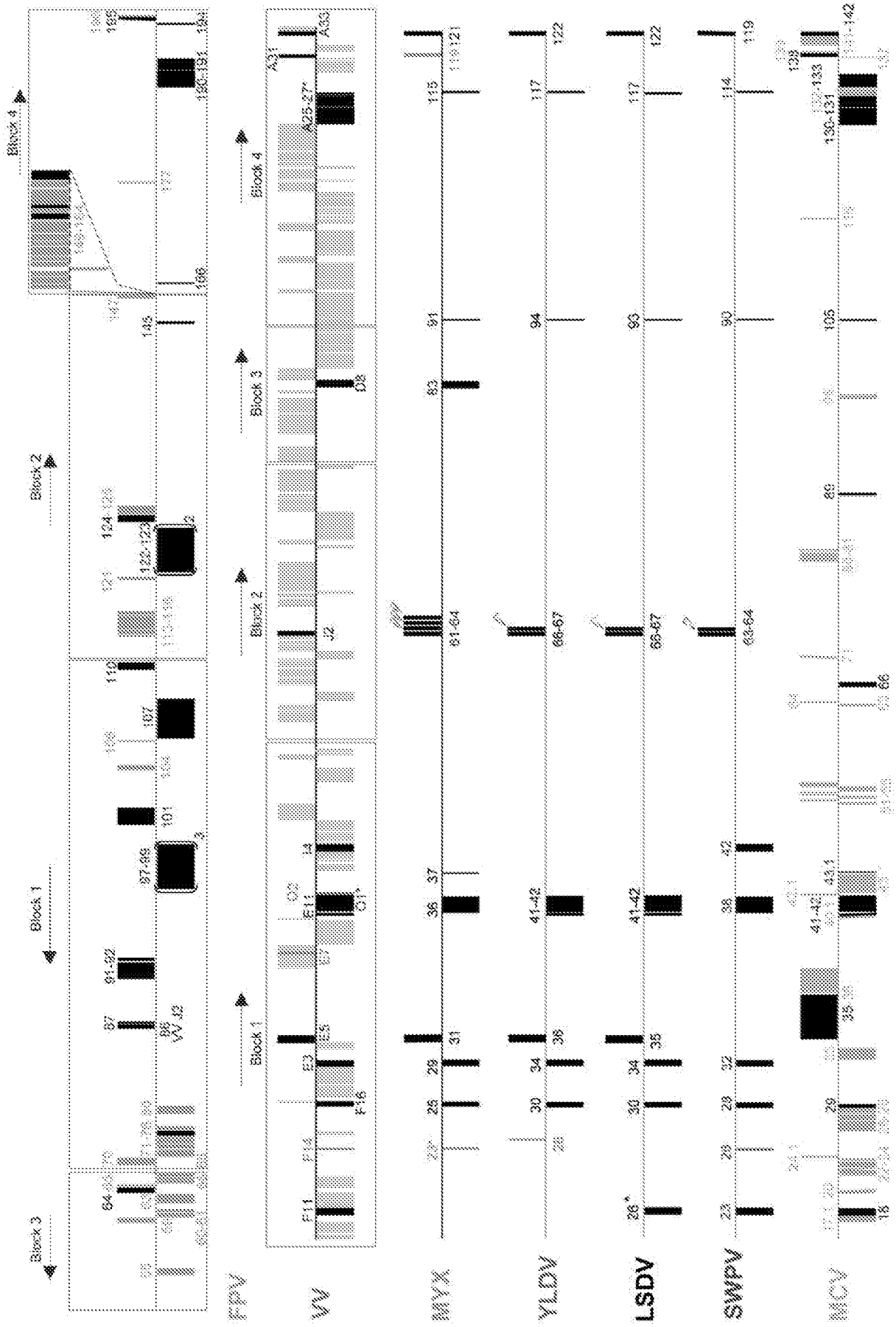
Block 1
Block 2
Block 3
Block 4
FPV
VV
MYXV
YLDV
LSDV
SWPV
MCV

Fig. 1 arrowheads) in the central region of the *Leporipoxvirus*, *Yatapoxvirus*, *Capripoxvirus* and *Suipoxvirus* genomes, but in the terminal region of OPVs. In some viruses, this gene has been triplicated. This indicates that the genera *Leporipoxvirus*, *Yatapoxvirus*, *Capripoxvirus* and *Suipoxvirus* form a subgroup that is distinct from the OPVs.

In contrast, the genomes of FPV (*Avipoxvirus*) and MCV (*Molluscipoxvirus*) are divergent from other ChPV genera and contain many unique genes. FPV also shows rearrangement of the conserved genes. Whereas all the other ChPV genera have a conserved gene order, blocks of FPV genes have been transposed and/or inverted. The blocks of VV genes that run left to right 1, 2, 3 and 4 are present in FPV in the order 3, 1, 2 and 4 with blocks 1 and 3 in inverted orientation (Afonso *et al.*, 2000). This observation suggests that the FPV genome is most divergent compared to the other ChPVs.

Fig. 1 does not include the EnPVs because these are too divergent from ChPVs by genome size, gene arrangement and gene sequence similarity. Previous work showed that EnPVs are also divergent amongst themselves (Afonso *et al.*, 1999; Bawden *et al.*, 2000), which suggests that EnPVs diverged from ChPVs before the ChPVs evolved into distinct genera. These results also agree with previous suggestions that the orthopteran and lepidopteran members of genus *B* of EnPV might be split into separate genera (Afonso *et al.*, 1999; Bawden *et al.*, 2000). The overall amino acid identity of the 17 proteins we used for phylogenetic analysis of ChPVs (next section) is between 26·0 % and 29·9 % when comparing either EnPV with any ChPV, and only 55 % between the two EnPVs. For comparison, this value is 98·7 % between two members of the OPV genus (VV-COP and VAR-BSH), 94·8 % between the two leporipoxviruses (MYX and *Shope fibroma virus* (SFV)] and ~94 % between different capripoxviruses (data not shown). Because EnPVs are too divergent to be compared to ChPVs, or even to provide a reliable root, they were omitted from further analysis.

Phylogenetic relationships

Previously, the evolutionary relationships of poxviruses had been investigated based largely on genome collinearity and the nucleotide or amino acid sequence alignment of a few genes or proteins. However, rigorous phylogenetic studies using DNA and protein sequences from multiple genes for each virus are lacking. To establish phylogenetic relationships for ChPVs, we have compared (i) multiple protein sequences that are conserved in all sequenced ChPVs, (ii) DNA and protein sequences from terminal regions of the genomes that are conserved in OPVs, and (iii) DNA sequences from the central 100 kb of OPV genomes.

To compare the different ChPVs we selected 17 out of the 49 proteins conserved in all poxviruses. These were aligned for one or more member of each genus, nine viruses in total: SWPV, YLDV, VV-COP, VAR-BSH, MYX, SFV, MCV, LSDV and FPV. The use of several protein sequences to produces a single tree is more likely to represent the species tree accurately than a tree constructed with any single sequence. Previously, phylogenetic trees for single OPV proteins gave variable topologies (Afonso *et al.*, 2002b; Gubser & Smith, 2002). Similarly, others reported inconsistent tree topologies using single genes from closely related species (Huelsenbeck & Bull, 1996). The proteins chosen for analysis (VV-COP E9L, I7L, I8R, G9R, J3R, J6R, H2R, H4L, H6R, D1R, D5R, D6R, D11L, D13L, A7L, A16L and A24R) were selected to represent enzymes that are essential for transcription or DNA replication, and structural components of new virions (Table 2). All selected proteins are of similar length in the different viruses and are well conserved. Alignments were made for individual proteins, these alignments were edited and the sequences were concatenated into a single file that was used to construct a maximum-likelihood tree (Methods; Fig. 2). A tree drawn using the neighbour-joining method gave similar data (data not shown). The branch structure of the maximum-likelihood tree is unequivocal.

The phylogenetic tree shows that ChPVs divide into four main groupings. The first two, the *Molluscipox* (MCV) and *Avipox* (FPV) genera, each group separately. FPV has 113 unique genes, presumably derived from its avian host(s), and has the largest ChPV genome (288 kb), another feature that distinguishes it from the other ChPV members. The FPV genome is not collinear with other ChPVs and within the central region contains gene families and individual genes related to cellular genes that are found within terminal regions of other ChPVs (Fig. 1) (Afonso *et al.*, 2000). The 17 FPV proteins examined show a mean amino acid identity of 61·3 % to 62·0 % with all other ChPVs but show no greater similarity to any specific genus. A distinctive feature of FPV is that it is the only ChPV not to contain a counterpart of

Fig. 1. Gene content of the central region of seven ChPV genomes. VV-COP is used as the reference against which other viruses are compared. Only the prototype of each genus is represented. For each genome, the genes (vertical bars) are located on the top strand when transcribed rightwards and on the bottom strand when transcribed leftwards. Genes that are members of the minimal gene complement (the 90 genes conserved in all ChPVs) are shown in grey for VV-COP. Genes conserved between two or more viruses from different genera (but are not one of the 90 conserved genes) are shown in black and genes that are unique to one genus are represented in the same colour as the virus name. Genes that are present in some members only of one specific genus, but are fragmented or absent in other members, are indicated with an asterisk. Block 1, counterparts of VV-COP *F9L–G4L*; block 2, counterparts of VV-COP *G5R–D2L*; block 3, counterparts of VV-COP *D3R–A1L*; block 4, counterparts of VV-COP *A2L–A34R*. The arrows illustrate the orientation of the blocks within the VV-COP and FPV genomes. Arrowheads indicate the positions of orthologues of VV-COP gene *C7L*.

Table 2. Minimal gene complement of chordopoxviruses

The 90 genes that are present in all sequenced ChPVs are listed together with their function where known. Genes are named after their VV-COP counterpart. Genes also present in the two EnPVs are highlighted. IMV, intracellular mature virus; IEV, intracellular enveloped virus; EEV, extracellular enveloped virus.

ORF	Putative function	ORF	Putative function
F9L	Unknown	H6R	DNA topoisomerase I
F10L	IMV serine-threonine protein kinase	H7R	Unknown
F12L	IEV protein	D1R	mRNA capping enzyme, large subunit
F13L	EEV protein/phospholipase	D2L	IMV core protein
F15L	Unknown	D3R	IMV core protein
F17R	IMV core phosphoprotein, VP11/DNA-binding protein	D4R	Uracil-DNA glycosylase
E1L	Poly(A) polymerase catalytic subunit	D5R	Nucleoside triphosphatase
E2L	Unknown	D6R	Early transcription factor small subunit, VETF-1
E4L	Poly(A) polymerase catalytic subunit, rpo30/VITF-1	D7R	RNA polymerase subunit rpo18
E6R	Unknown	D9R	29 kDa mutT-like protein
E8R	Unknown	D10R	29 kDa mutT-like protein, negative regulator of gene expression
F9L	DNA polymerase	D11L	Nucleoside triphosphate phosphohydrolase I
E10R	IMV membrane-associated protein	D12L	mRNA capping enzyme small subunit, intermediate transcription factor, VITF
I1L	IMV core/DNA-binding protein	D13L	IMV protein, rifampicin resistance
I2L	Unknown	A1L	Late transcription factor/VLTF-2
I3L	Phosphoprotein, binds ssDNA	A2L	Late transcription factor/VLTF-3
I5L	IMV structural protein, VP13K	A2·5L	Thioredoxin-like protein
I6L	Unknown	A3L	IMV major core protein, P4b
I7L	IMV core protein	A4L	IMV core protein
I8R	Nucleoside triphosphate phosphohydrolase II, RNA helicase, NTPase	A5R	RNA polymerase subunit rpo19
G1L	Metallo-endoproteinase/virion morphogenesis	A6L	Unknown
G2R	Late transcription/IBT-dependent protein	A7L	Early transcription factor large subunit, VETF
G3L	Unknown	A8R	Intermediate transcription factor, VITF-3
G4L	Glutaredoxin 2, membrane protein, virion morphogenesis	A9L	IMV protein, role in morphogenesis
G5R	Unknown	A10L	IMV major core protein P4a
G5·5R	RNA polymerase subunit rpo7	A11R	Unknown
G6R	Unknown	A12L	IMV core protein
G7L	IMV core protein, VP16K	A13L	IMV membrane-associated protein/p8
G8R	Late transcription factor, VLTF-1	A14L	IMV protein, p16
G9R	Myristyl protein	A14·5L	IMV protein
L1R	Myristylated IMV protein	A15L	Unknown
L2R	Unknown	A16L	Myristyl protein
L3L	Unknown	A17L	IMV membrane protein, morphogenesis factor
L4R	IMV core protein VP8, DNA and RNA-binding protein	A18R	DNA helicase, DNA-dependent ATPase, transcript release factor
L5R	Unknown	A19L	Unknown
J1R	Dimeric virion protein	A20R	DNA polymerase processivity factor
J3R	Poly(A) polymerase stimulatory subunit, VP39	A21L	Unknown
J4R	RNA polymerase subunit rpo22	A22R	Holliday junction resolvase
J5L	Unknown	A23R	Intermediate transcription factor, VITF-3
J6R	RNA polymerase subunit rpo147	A24R	RNA polymerase subunit rpo132
H1L	Tyrosine-serine phosphatase, virion maturation	A28L	Unknown
H2R	Unknown	A29L	RNA polymerase subunit rpo35
H3L	Immunodominant IMV envelope protein p35	A30L	Unknown
H4L	RNA polymerase-associated transcription specificity factor, RAP94	A32L	ATP- and GTP-binding motif A, DNA packaging
H5R	Late transcription factor, VLTF-4	A34R	EEV glycoprotein

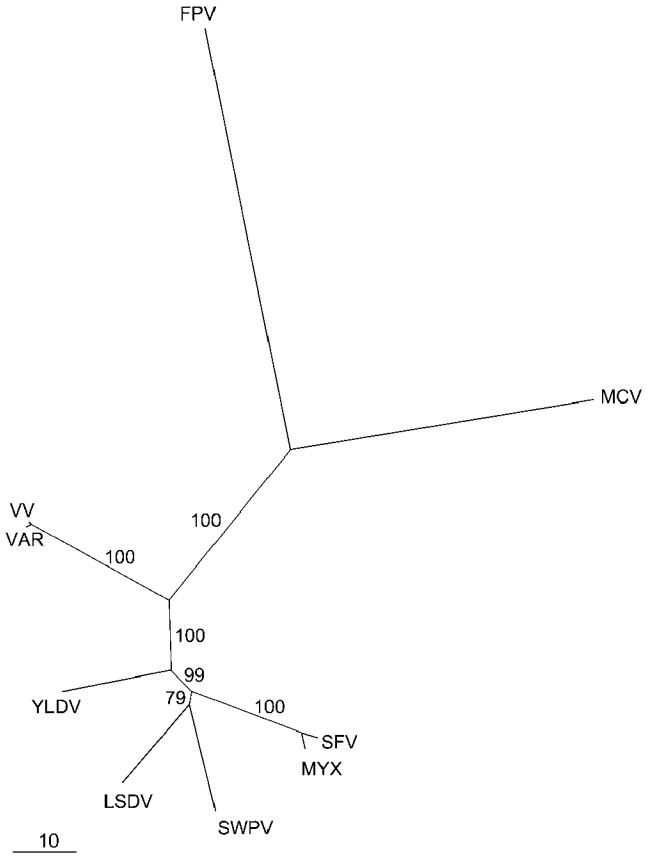

Fig. 2. Phylogenetic analysis of ChPVs. The amino acid sequences of 17 poxvirus proteins (VV-COP E9L, I7L, I8R, G9R, J3R, J6R, H2R, H4L, H6R, D1R, D5R, D6R, D11L, D13L, A7L, A16L and A24R) were aligned as described in Methods. The maximum-likelihood tree was obtained using the program ProML from the PHYLIP package version 3.0 (JTT model with gamma distribution) and is shown in an unrooted format. The bootstrap values from 1000 replica samplings and the divergence scale (substitutions per site) are indicated.

VV-COP gene *A33R*, which encodes an extracellular enveloped virus (EEV) envelope protein.

After the eradication of smallpox, MCV remains the only endemic human-specific poxvirus and it is divergent from other ChPVs. MCV is well adapted to humans (it survives long term and causes little morbidity) and this is reflected by 70 unique proteins (including several immunomodulators) and the lack of most of the immunomodulators encoded by other poxviruses (Senkevich *et al.*, 1996). Previous phylogenetic studies carried out using single MCV proteins resulted in different tree topologies depending on the gene and the method of tree construction, with MCV grouping individually in most cases but also together with FPV (Senkevich *et al.*, 1996). Data presented in Fig. 1 show that MCV and FPV are distinct ChPVs that have diverged from other ChPVs long ago. Like FPV, the conserved MCV proteins show a modest percentage amino acid identity with other ChPVs (range 61·7 % to 63·4 %) and MCV is no closer to any specific ChPV genus. MCV is the only ChPV not to contain a counterpart of VV-COP gene *J2R* (thymidine kinase).

The third and largest cluster of ChPVs includes the *Yatapoxvirus* (YLDV), *Capripoxvirus* (LSDV), *Suipoxvirus* (SWPV) and *Leporipoxvirus* (SFV and MYX) genera. Within this group, SFV and MYX, which cluster strongly together, also group with SWPV and LSDV, whereas YLDV is slightly more divergent. The genomes of all these viruses are relatively well conserved in gene content, gene arrangement (Fig. 1) and amino acid identity (data not shown). Notably, unlike OPVs, these viruses all contain at least one counterpart of VV gene *C7L* within the central region of their genomes (Fig. 1, arrowheads) between counterparts of VV-COP genes *J2R* and *J3R*. For these four ChPV genera, the overall amino acid identity is highest between SWPV and LSDV (79·2 %). This is consistent with the tree topology (Fig. 2) and suggests that SWPV and LSDV have evolved from a common ancestor.

The fourth ChPV group is genus OPV, illustrated by VV-COP and VAR-BSH, which group together tightly and separately from other ChPVs. The scale of the phylogenetic tree shows how closely related these viruses are compared to, for instance, the different leporipoxviruses and suggests that this group of viruses diverged more recently than members of other ChPV genera. Another feature that distinguishes the OPVs from other ChPVs is the presence of genes equivalent to VV-COP *F14L*, *E7L* and *O2L* within the central conserved region. ChPVs from outside the OPV genus lack these genes.

In summary, the comparison of poxviruses from different ChPV genera with each other using two different computational methods gave a robust phylogenetic tree. The only ChPV genus not represented here is *Parapoxvirus*, for which a complete genome sequence is awaited. The OPV genus is now considered in more detail.

Orthopoxvirus phylogeny

OPVs are the most intensively studied poxviruses. The reasons for this are largely historical: smallpox, caused by VAR, used to be a very serious disease of mankind; CPV is thought to have been used by Jenner in 1798 as the first human vaccine; and VV is the smallpox vaccine that was used in the modern era. Currently, there are 12 complete OPV genome sequences from 6 species (VAR, VV, CPV, MPV, ECT, CMPV) (Table 1). The origin and evolutionary relationships of these viruses are ill-defined, although it was demonstrated recently that CMPV-CMS and VAR are closely related (Gubser & Smith, 2002).

To compare the phylogeny of OPVs we selected genes that are conserved in the terminal genome regions of these viruses, where genes have greater divergence between species. Within the terminal regions of 10 sequenced OPVs, only 12 out of ~100 genes are present in every virus and this is due in part to mutations causing disruption of several genes in different

OPVs. The conserved genes are VV-COP *C7L* (host range function; Perkus *et al.*, 1990), *C6L* (unknown), *N1L* (intracellular virulence factor; Bartlett *et al.*, 2002), *K2L* (serine proteinase inhibitor, SPI-3; Law & Smith, 1992), *F2L* (dUTPase; McGeoch, 1990), *F4L* (ribonucleotide reductase, small subunit; Slabaugh *et al.*, 1988), *F6L* (unknown), *F8L* (unknown), *A56R* (the haemagglutinin glycoprotein that forms part of the extracellular enveloped virus outer envelope; Shida, 1986), *B1R* (protein kinase; Banham & Smith, 1992), *B5R* (EEV glycoprotein; Engelstad *et al.*, 1992) and *B15R* (unknown). These genes are known or likely to have an important function.

The proteins encoded by the selected genes were aligned and used to construct a maximum-likelihood tree (Methods, Fig. 3). Several facts may be deduced from the tree. First, the close relationship of CMPV and VAR is confirmed. The three strains of VAR each cluster together, as do the two strains of CMPV, and the VAR and CMPV clusters are more closely related to each other than to any other OPV species. Second, CPV-BR, MPV and ECT do not group closely with other OPVs. Lastly, although the two VV strains cluster closely together, the two CPV strains do not and they show

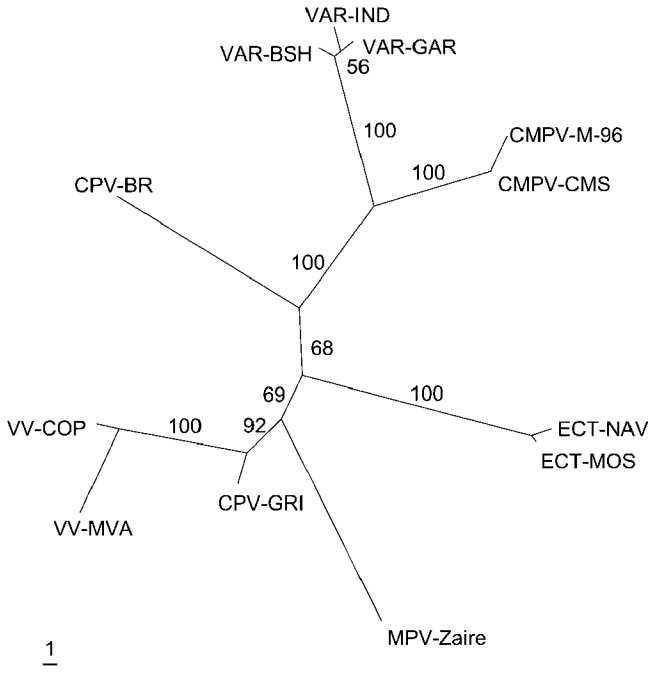

Fig. 3. Phylogenetic tree of 12 OPVs obtained by the maximum-likelihood method using protein sequences. The amino acid sequences of 12 OPV proteins (VV-COP C6L, C7L, N1L, K2L, F2L, F4L, F6L, F8L, A56R, B1R, B5R, B15R) encoded in the terminal regions of the genomes were aligned as described in Methods. The maximum-likelihood tree was obtained using the program ProML from the PHYLIP package version 3.0 (JTT model with gamma distribution) and is shown in an unrooted format. The bootstrap values from 1000 replica samplings and the divergence scale (substitutions per site) are indicated.

remarkable divergence for two strains of the same virus species. It will be interesting to determine if other CPVs group predominantly with CPV-BR or GRI-90 or even form further divergent groups. These results suggest that classification of CPV as a single species within the genus OPV needs reconsideration.

Next, we examined the DNA sequences of these genes from eight OPVs (Fig. 4a). Four OPVs were omitted because the extra strains of VAR, CMPV and ECT cluster closely with other members of their species (Fig. 3). This analysis confirmed the relationships observed for the protein sequences in Fig. 3. CMPV and VAR are closely related, whereas CPV-BR, ECT and MPV are divergent. As in Fig. 3, the two strains of CPV are sufficiently divergent from one another to justify being classified as independent species. We also analysed if the relationships of genes from different ends of the genome were the same (Fig. 4b, c). These trees confirm the overall relationships, and show that CPV-GRI-90 is always closest to VV and divergent from CPV-BR. However, CPV-BR and ECT-NAV group differently in the two cases. Genes from the left end of the genome place CPV-BR closer to the VV subgroup, and ECT-NAV closer to VAR and CMPV (Fig. 4b). Conversely, genes at the right end show the opposite relationships (Fig. 4c). These results might suggest that recombination events have occurred in these viruses.

To investigate this further, the nucleotide sequences of CPV-BR or ECT-NAV genes from the left or right end of the genome were compared using SimPlot with the equivalent genes from either CMPV-CMS and VAR-BSH or VV-MVA and VV-COP. Consistent with Fig. 4(b, c), genes from the left end of the CPV-BR genome are closer to the VV strains, whereas genes from the right end are more related to the CMPV/VAR subgroup (Fig. 5a). In contrast, genes from the left end of ECT-NAV show a similar relationship to each subgroup, whereas at the right end genes are closer overall to the VV subgroup (Fig. 5b). These results confirm the different topologies of the trees shown in Fig. 4(b, c), and also provide evidence of recombination within the right end of the genomes of CPV-BR and ECT-NAV.

Finally, we analysed the relatedness of the central ~100 kb of the OPV genomes. This region shows >90 % nucleotide identity between all OPVs and encodes all genes conserved throughout the ChPV subfamily. Remarkably, the maximum difference in length for this region was 382 nucleotides between CPV-BR (longest) and MPV (shortest), and CMPV-CMS and VAR-BSH differ in length by only 82 nucleotides. Most differences were intergenic caused by repeated oligonucleotides in one virus or another. Similar repeated oligonucleotides had been reported between VV-COP and VV-WR (Smith *et al.*, 1991) and VV-WR and VAR-Harvey (Aguado *et al.*, 1992). An alignment of this region is presented as supplementary data (JGV Online: http://vir.sgmjournals.org).

From this alignment, a distance matrix was constructed and is shown in Table 3. As expected the genetic distances

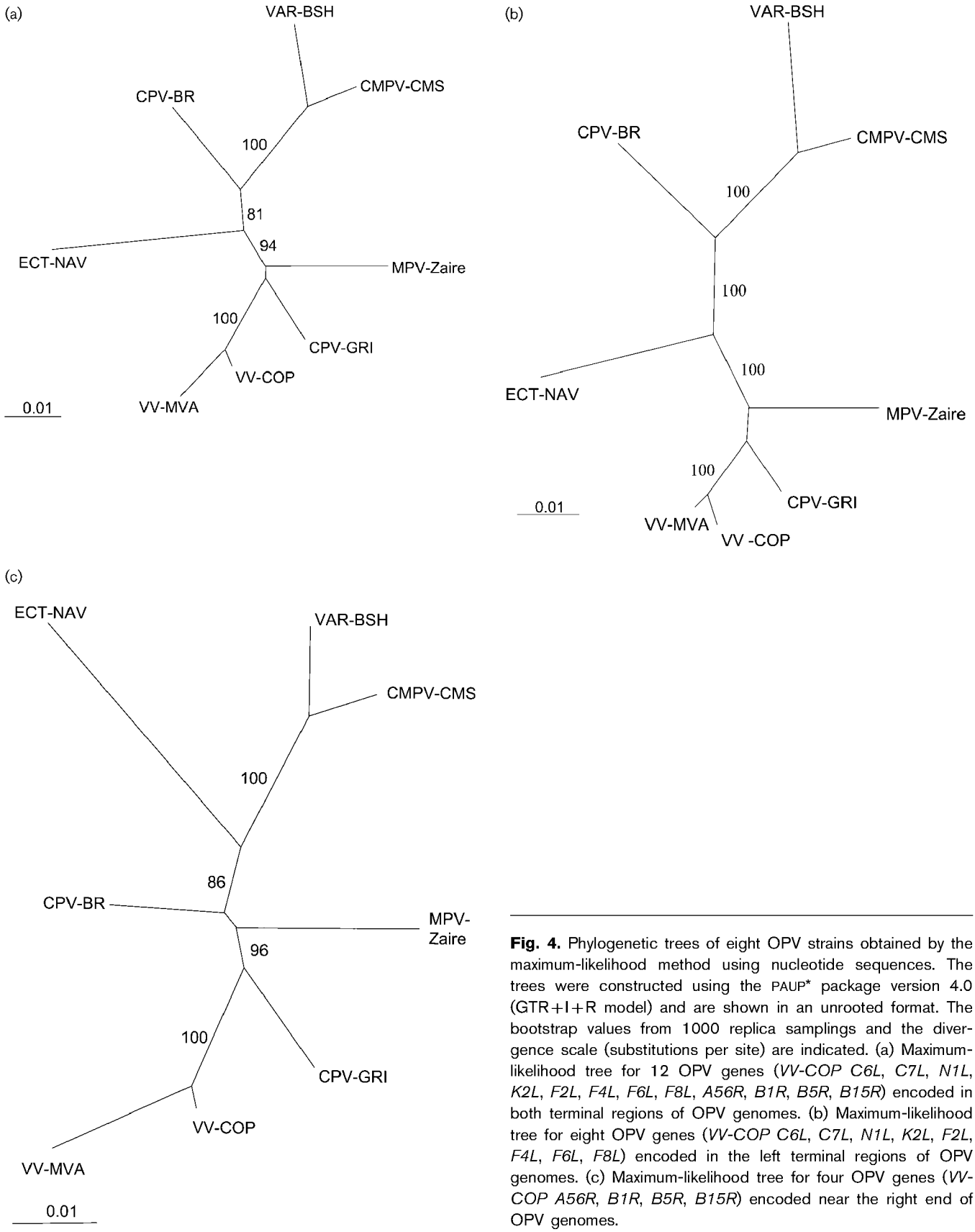

Fig. 4. Phylogenetic trees of eight OPV strains obtained by the maximum-likelihood method using nucleotide sequences. The trees were constructed using the PAUP* package version 4.0 (GTR+I+R model) and are shown in an unrooted format. The bootstrap values from 1000 replica samplings and the divergence scale (substitutions per site) are indicated. (a) Maximum-likelihood tree for 12 OPV genes (*VV-COP C6L*, *C7L*, *N1L*, *K2L*, *F2L*, *F4L*, *F6L*, *F8L*, *A56R*, *B1R*, *B5R*, *B15R*) encoded in both terminal regions of OPV genomes. (b) Maximum-likelihood tree for eight OPV genes (*VV-COP C6L*, *C7L*, *N1L*, *K2L*, *F2L*, *F4L*, *F6L*, *F8L*) encoded in the left terminal regions of OPV genomes. (c) Maximum-likelihood tree for four OPV genes (*VV-COP A56R*, *B1R*, *B5R*, *B15R*) encoded near the right end of OPV genomes.

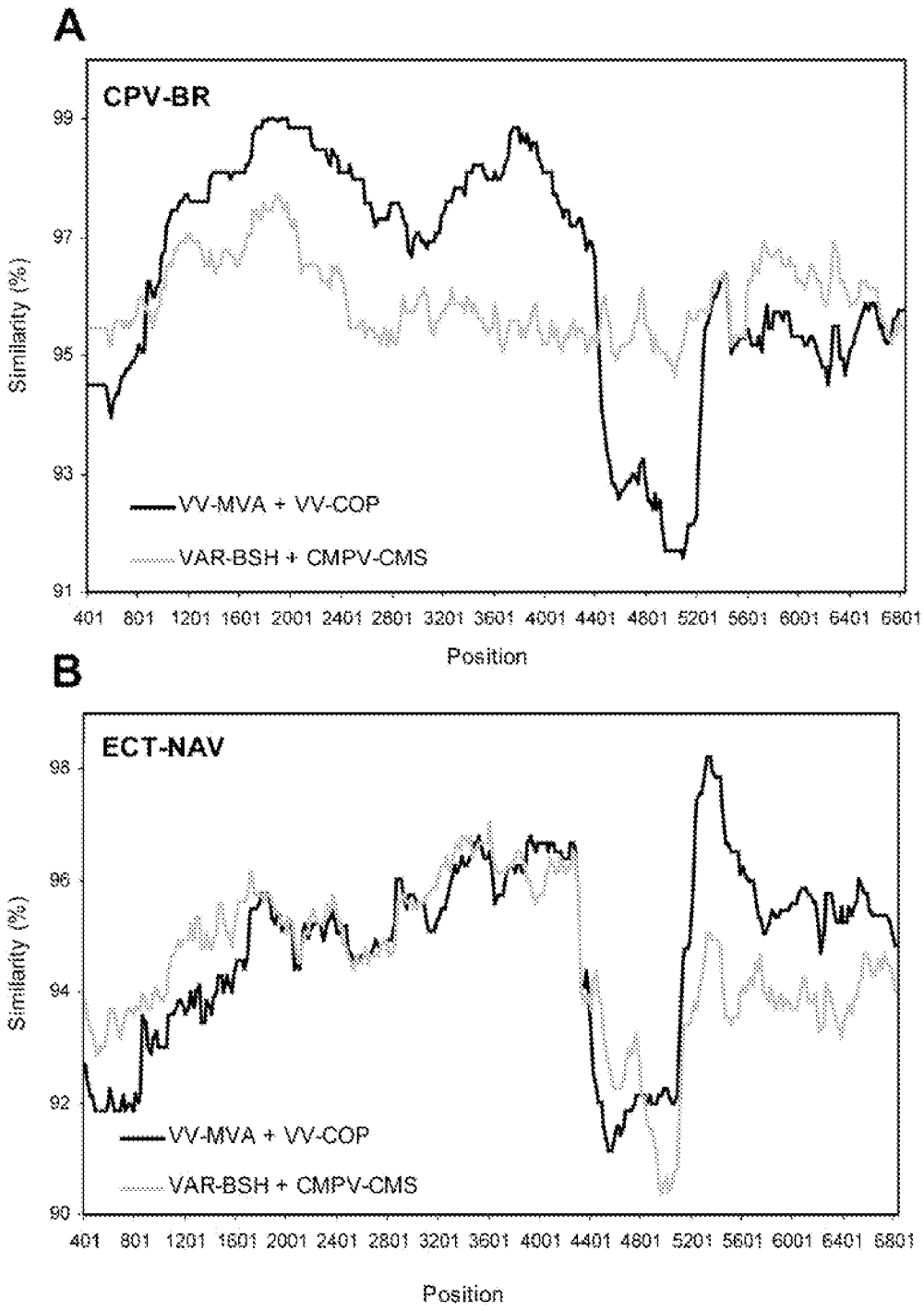

Fig. 5. Evidence for recombination between OPV genomes. The percent nucleotide similarity between each query sequence CPV-BR (A) and ECT-NAV (B) and the VAR-BSH/CMPV-CMS and the VV-MVA/VV-COP groups of sequences is represented by a grey and a black line, respectively. A sliding window of 800 bases was used along the nucleotide alignment, with an increment of 20 bases. In this alignment, the first 4170 and last 3063 nucleotides are from the left or right ends of the genome, respectively. The similarity values between the query and the reference sequences were computed according to the F84 model of evolution, with a transition/transversion ratio of 1·95.

between OPVs are low (0·0150–0·0354) compared to the genetic distance for the same region of SFV and MYX, which was eightfold greater than between CMPV and VAR (Gubser & Smith, 2002). This suggests that OPVs have diverged more recently than the leporipoxviruses from their common ancestor. When comparing different OPV species, the

Table 3. DNA distance matrix

DNA sequences between counterparts of VV-COP genes *F9L* and *A24R* were aligned using the program CLUSTALW version 1.8 (Thompson *et al.*, 1994. See supplementary data) and a DNA distance matrix was constructed using the program DNADIST from the PHYLIP package version 3.6 (alpha2) (Felsenstein, 1989) as described in Methods.

		VV			VAR			CMPV		MPV	ECT	CP
		TT	COP	MVA	IND	BSH	GAR	CMS	M-96	Zaire	NAV	BR
VV	TT											
	COP	0.0058										
	MVA	0.0076	0.0060									
VAR	IND	0.0268	0.0259	0.0255								
	BSH	0.0268	0.0259	0.0254	0.0012							
	GAR	0.0267	0.0258	0.0253	0.0036	0.0030						
CMPV	CMS	0.0222	0.0213	0.0209	0.0154	0.0150	0.0151					
	M-96	0.0223	0.0214	0.0209	0.0155	0.0151	0.0152	0.0002				
MPV	Zaire	0.0216	0.0205	0.0204	0.0307	0.0305	0.0304	0.0261	0.0262			
ECT	NAV	0.0285	0.0274	0.0271	0.0354	0.0351	0.0352	0.0313	0.0315	0.0298		
CPV	BR	0.0249	0.0238	0.0237	0.0329	0.0326	0.0325	0.0285	0.0286	0.0269	0.0281	

genetic distance was lowest between strains of CMPV and VAR (0·0151–0·0154), and highest between ECT and VAR (0·0352–0·0354) (Table 3).

DISCUSSION

Comparison of 26 sequenced poxviruses has identified 90 genes that are conserved in all ChPVs and 49 genes that are conserved in all poxviruses. These numbers are in agreement with Upton *et al.* (2003). All the conserved ChPVs genes lie within the central region of the genome.

The phylogenetic relationships of the sequenced poxviruses were examined. The genome organization, and percentage amino acid sequence identities, showed that the two sequenced EnPVs are distinct from ChPVs and quite divergent from each other so that they might be classified in separate genera (Afonso *et al.*, 1999; Bawden *et al.*, 2000). Within the ChPVs, the most divergent virus is FPV (genus *Avipoxvirus*) followed by MCV (genus *Molluscipoxvirus*). This overall conclusion is reached by comparison of the size of these genomes, the number of unique genes, the gene arrangement (Fig. 1) and phylogenetic analysis of the amino acid sequences of 17 conserved proteins (Fig. 2). Avipoxviruses are the only ChPVs to infect birds and MCV is a strictly human pathogen; both viruses have evolved unique immunomodulatory proteins that enable them to counteract the immune system of their hosts.

The other ChPVs show two clusters: the first includes the genera *Leporipoxvirus*, *Capripoxvirus*, *Suipoxvirus* and *Yatapoxvirus*, within which SWPV and LSDV share a common ancestor; the second is genus OPV. The first group of viruses have smaller genomes [range 144 575 (YLDV) to 161 774 (MYX); Table 1] and few unique genes in the central region of the genome (Fig. 1). These genomes also all have the orthologue of the VV-COP gene *C7L* within the central region of the genome, whereas in OPVs this is present near the left end. The relatively small size (139 kb) of the *Orf virus* genome (Mercer *et al.*, 1987) suggests genus *Parapoxvirus* might group within this cluster.

The OPVs represent a closely related group of viruses with larger genomes [177 923 (VV-MVA) to 224 501 (CPV-BR)]. Note that VV-MVA has lost about 30 kb compared to the parental Ankara strain (Meyer *et al.*, 1991).

Overall, the phylogenetic analyses of poxvirus protein sequences give relationships between genera (Fig. 2) that are consistent with relationships deduced from comparisons of genome organization and gene content (Fig. 1).

A more detailed analysis of OPVs revealed that the central regions of these genomes are very similar. Here the genes are collinear and over ~100 kb CMPV-CMS and VAR-BSH differ in length by only 82 nucleotides. Because of the high degree of similarity of genes and proteins from this region of OPVs, we compared the phylogenetic relationships of these viruses by using the DNA and protein sequences of genes from the terminal regions of the genome (Figs 3–5). These analyses established phylogenetic relationships but also indicated that these viruses have undergone recombination during their evolution. Data presented show that CMPV and VAR are closely related species, as reported previously (Gubser & Smith, 2002), but CPV-BR, MPV and ECT are divergent and do not group closely with other OPVs. For MPV, this is despite it causing a disease in man similar to smallpox. MPV occurs naturally in western and central Africa but is poorly transmitted from person to person and human infections tend to be limited local outbreaks. It has been proposed that rodents are the natural host for MPV (Fenner *et al.*, 1988).

The origin of the most extensively studied OPV, VV, is obscure. If Jenner used CPV as the first smallpox vaccine in 1796, sometime between then and 1939 when A. W. Downie reported that the available smallpox vaccine strains were a

distinct OPV that became known as VV (Downie, 1939a, b), CPV was replaced by VV as the smallpox vaccine. This probably had occurred by the late nineteenth century because the smallpox vaccine taken to the USA in 1856 and which became the New York City Board of Health Vaccine is VV not CPV. Similarly, pathologists who studied cells infected by smallpox vaccines used in the late nineteenth century reported the eosinophilic B type inclusion bodies made by VV and CPV, but failed to report the much more obvious A type inclusion bodies that are made by CPV but not VV. Therefore, by this time VV was probably already used as the smallpox vaccine. The possible origin of VV was discussed by Baxby (1981). He proposed VV was a distinct OPV species that was isolated from a species in which it was no longer endemic. Horsepox was one possibility. In support of this proposal, early vaccinators took vaccine from horses when the supply of CPV (a relatively rare disease) was scarce and one strain of VV (Ankara) was isolated from a horse. The recent demonstration that the VV-WR interferon-γ receptor binds and neutralizes equine interferon-γ (Symons *et al.*, 2002) is also consistent with this proposal. However, given the broad host range of VV and the broad species specificity of the VV interferon-γ receptor, these observations might be interpreted only as VV being a zoonosis in horses and that its natural host lies elsewhere. Phylogenetic comparisons indicate VV is not derived recently from either VAR or CPV but that it is closer to CPV-GRI-90 than CPV-BR.

An interesting feature of OPV genomes is the presence of many genes that are intact in one virus but fragmented in another. Comparison between OPV genomic sequences reveals that fragmented ORFs (i) are located mainly within 50 kbp of either terminus, (ii) represent 33/206 ORFs (16 %) in CMPV (Gubser & Smith, 2002) and (iii) are fewest in CPV (Shchelkunov *et al.*, 1998). Many of these gene fragments are unlikely to encode functional protein, so their retention is surprising. This might reflect the relative stability of OPV genomes and the apparent lack of a stringent packaging limit on poxvirus DNA (Smith & Moss, 1983; Perkus *et al.*, 1991). Another interpretation is that some OPVs are relatively recent pathogens of their respective hosts and have diverged from an ancestral virus recently in an evolutionary timescale, with disruption of some genes accompanying their divergence. The much closer relationship between OPVs than between leporipoxviruses is consistent with this interpretation (Fig. 2, Table 3). With time these viruses might have lost some of these non-essential gene fragments. In contrast to OPVs, within the leporipoxviruses SFV contains eight fragmented genes compared to MYX, but MYX contains only one fragmented gene compared to SFV (Cameron *et al.*, 1999; Willer *et al.*, 1999).

Finally, the two CPV strains are sufficiently divergent to justify their reclassification as different OPV species and it will be interesting to determine how other CPV strains isolated from different geographical locations compare with the two strains analysed to date. CPV-GRI-90 was proposed as a possible ancestral virus for OPVs because its genes in the terminal genome regions are mostly complete (Shchelkunov *et al.*, 1998), whereas other OPVs possess broken fragments of these genes. By this criterion, CPV-BR also might be considered close to a possible ancestral virus.

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